Genetics of asthma and atopy

Rijksuniversiteit Groningen

Genetics of asthma and atopy

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"The human genome underlies the fundamental unity of
all members of the human family, as well as the
recognition of their inherent dignity and diversity.
In a symbolic sense, it is the heritage of humanity."

Universal Declaration on the Human Genome and Human Rights

Voor *Annouk*

Paranimfen

H. Jongepier
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Contents

Introduction
The genetics of asthma and atopy

The central subject of this thesis is the genetics of asthma and atopy. This thesis comprises different aspects of genetic studies of asthma and atopy, from the definition of the asthma phenotype to linkage, association, and functional studies. In this introduction, we will first give definitions and then discuss some clinical, epidemiological, physiological, and immunopathological aspects of asthma and atopy. This will be followed by an introduction into genetic research and a discussion of different methods to investigate the genetics of complex diseases. Finally, the research questions that have guided our research are formulated.

Atopy and asthma

Atopy comprises all IgE mediated diseases, such as asthma, allergic rhinitis and atopic dermatitis. Individuals with atopy have a genetic predisposition to produce IgE antibodies against common environmental allergens.[1]

Asthma is currently defined as a "chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular mast cells, eosinophils, T-lymphocytes, macrophages, neutrophils and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial responsiveness to a variety of stimuli." (National Heart Lung and Blood Institute, 1995).[2] This definition combines clinical characteristics (e.g. wheezing, breathlessness) and physiological characteristics of asthma (variable airway obstruction, bronchial hyperresponsiveness) with its underlying pathology, which is the inflammation of the airway walls.

Asthma and atopy have a close interrelation. Atopy often precedes asthma and it increases the risk of developing asthma 10 – 20 fold.[3] The initial sensitisation to environmental allergens occurs typically in childhood. A subset (25-30%) of those sensitised to environmental allergens develops asthma.[4] Both the level of sensitisation, expressed as the number of positive skin tests, and the type of sensitisation are important for asthma development.[5] The association of asthma with sensitisation to a particular allergen appears to depend on the geographical distribution of the allergen. In the Netherlands, the major allergen is derived from house dust mites.[6] In other countries with a low prevalence of house dust mites, other allergens such as cat, dog, cockroach and the mould alternaria are important.[7] In childhood, virtually all patients with asthma have atopy, whereas in adulthood also non-atopic asthma, so-called intrinsic asthma, can be found. Patients with intrinsic asthma present typically in adulthood, have variable airways obstruction, and may not have complete reversibility of airways obstruction.[8] In the study presented in this thesis, 15 % of the patients with asthma of whom we studied their families did not have atopy. It is unknown if intrinsic asthma is a separate condition with a distinct genetic susceptibility.[9]

Clinical expression of atopy

Patients with asthma have in general a history of episodic breathlessness together with cough and wheeze. In asthma, symptoms such as cough and wheezing occur frequently at night.[10] Patients may also experience symptom free intervals. The prevalence of symptoms in children with asthma decreases into adulthood. For example, in a study of a cohort of 119 allergic asthmatic children, virtually all children reported wheeze in childhood. However, only 18 % still reported wheeze when followed up at age 32- 42.[11]

Allergic rhinitis is defined as an inflammation of the lining of the nose, characterised by one or more of the following symptoms: nasal congestion, rhinorrhoea, sneezing and itching.[12] In seasonal allergic rhinitis, symptoms exist in the pollen season, whereas in perennial allergic rhinitis symptoms are chronic and persistent throughout the year.
Atopic dermatitis, also called eczema, is a chronically relapsing inflammatory skin disease. It is characterised by itching, leading to scratching and excoriations. In addition, dryness of the skin is a typical finding in atopic dermatitis. In it first stage (age 0-3 years), typically the scalp and cheeks are affected by a pruritic erythema with papules, vesicles, exudation and excoriations. Later on in childhood, typical sites of atopic dermatitis are the flexural sides of elbows and knees. It has been reported that as many as 50 - 75 % of patients with eczema in early childhood will develop allergic rhinitis or asthma.[13] In this thesis we will focus on respiratory atopic diseases, in particular asthma.

Epidemiology

The prevalence of atopic diseases in childhood in Western populations is high. There are large worldwide differences in its prevalence. In the ISAAC study, the prevalence of atopy was investigated in 56 countries in children aged 13-14 years. The 12-month prevalence of symptoms of asthma ranged from 1.6 to 36.8 %, compared to allergic rhino-conjunctivitis (1.4 - 39.7 %) and atopic dermatitis (0.3 - 20.5%). Western countries, such as the United Kingdom, USA Australia and Canada were found among the countries with the highest prevalence.[14] Data for the Netherlands are available through the European Respiratory Health Survey, a random population study of adults aged 20 - 48 years. Three regions in the Netherlands (Groningen, Bergen op Zoom and Geleen) were investigated. Prevalences of wheeze were reported to be 18.2%, 18.9%, and 18.8%; and attacks of asthma 3.2%, 3.1%, and 1.5%, respectively.[15] The prevalence of atopy as assessed by specific IgE to four aeroallergens was 36.1 % in the Netherlands in this study. [6]
Prevalence rates depend a.o. on the definitions that have been used to assess asthma and atopy. For asthma, a doctor's diagnosis, asthma symptoms or definitions based on bronchial hyperresponsiveness have been used. For example, in Australia and New Zealand, prevalence of self reported asthma has been reported to be 16.3 % (adults, Busselton), whereas the prevalence of wheeze was 28.8 % in the same population.[16] A difference between the presence of bronchial hyperresponsiveness and a doctor's diagnosis of asthma was observed in first and second degree relatives of 92 patients with asthma in our family study. 113 out of 320 children had bronchial hyperresponsiveness to histamine. Of these 113 children, 33 (29 %) had a prior doctor's diagnosis of asthma. Of the 207 children without bronchial hyperresponsiveness to histamine,[15] 7.2 % had a prior doctor's diagnosis of asthma.[17]
Evidence is accumulating that the prevalence of asthma has risen over the last 20 – 30 years.[18] This is paralleled by a rise in atopy, as measured by a positive skin prick test, over the last decades.[19] It is thought that only a part of

the rise in asthma could be explained by atopy. It remains to be established what drives the increased prevalence of asthma.[20]
Different environmental factors have been investigated to explain the rise in prevalence of atopy and asthma.[19] For atopy, provoking factors include exposure to allergens, current or in early life, smoking, and air pollution, whereas possible protective factors include infant breast feeding, infections in early life, living at a farm, and exposure to lipopolysacherides. For asthma, these factors have also been investigated, together with a possible role of diet.[16, 21] Current data suggest that allergen exposure early in life is associated with sensitisation, but not with wheeze, asthma and bronchial hyperresponsiveness at age 7.[22] An important hypothesis that may explain epidemiological observations of inverse associations between childhood infections and atopy is the hygiene hypothesis. This hypothesis was originally proposed in 1989 by Strachan. He proposed that 'allergic diseases could be prevented by infections in early childhood, transmitted by unhygienic contact with older siblings, or acquired prenatally'.[23] This hypothesis was consistent with epidemiological observations of inverse relationships of atopic parameters (hay fever, skin prick tests) with childhood infections, a higher number of (older) siblings, and vaccination status.[23] In addition, high exposure to lipopolysacherides (LPS) in house dust was inversely related to the prevalence of sensitisation in a study of children aged 9 - 24 months.[24] One receptor through which LPS may exert its function is CD14. Association of a promoter variant in this gene with severity of atopy has been described in a US population of school children.[25] This finding was confirmed in a case-control study in the Dutch population as described in chapter 8 of this thesis. Immunological support for this hypothesis came from the observation that bacterial products, such as lipopolysacherides are inducers of a non-allergic (Th1-type) immunity. This will be discussed in more detail in the section on immunology.

Lung function and physiology

Two functional alterations are typically associated with asthma: variable airway obstruction and bronchial hyperresponsiveness.[26] In healthy individuals, airway size depends on several factors: its relaxed dimension, its elastic properties and the tone of the smooth muscle in its wall. In individuals with asthma, several factors contribute to airways narrowing, such as smooth muscle contraction, airway wall thickening, oedema and increased secretion of mucus.[26] In the lung function laboratory, airway obstruction can be demonstrated by forced expiratory tests or measurements of airway resistance and conductance. At home, serial peak expiratory flow measurements may indicate variable airway obstruction.
Bronchial hyperresponsiveness (BHR) is defined by a heightened sensitivity of the bronchi in response to non-specific stimuli. There are different types of stimuli to which patients with asthma show elevated responsiveness of the airways: parasympathicomimetic agents (e.g. methacholine),

β-receptor antagonists (propranolol), mediators (e.g. histamine, serotonin, adenosine), osmotic/physical stimuli (e.g. hypotonic or hypertonic saline, cold air) and finally air pollutants (e.g. ozone). Stimuli that evoke BHR can also be distinguished in stimuli that act directly on receptors of airway smooth muscle cells and indirect stimuli that are thought to act by stimulation of cellular and neurogenic pathways (adenosine-3- monophasphate, cold air).[27] In general, the concentration of the agent that after inhalation causes a 20% drop in FEV_1 (forced expiratory volume in 1 second) is lower than in healthy individuals. In addition, it appears that the type of the response is also different between asthmatic and normal airways. In asthma, airway narrowing can be progressive with higher doses of the challenge agent, whereas airways of normal individuals reach a plateau phase of maximum airway narrowing.[26]

Pathology and immunology

A recent review discussed the immunopathological findings in the central and peripheral airways found to be more or less characteristic of asthma. These constituted of "denudation of airway epithelium, deposition of collagen beneath the basement membrane, mast cell degranulation, and infiltration of the airway by lymphocytes and eosinophils".[28] An important feature seen in asthma is remodelling of the airways walls. Airway remodelling comprises all alterations in structural cells and tissues in the asthmatic when compared to the normal airway.[29] These include thickening of all components of the airway wall, subepithelial fibrosis (thickening of the basement membrane), increase myocyte muscle mass, myofibroblast hyperplasia and mucus metaplasia.[29] Mediators that may be important in airway remodelling are not completely known, but possibly include TGF-ß family proteins and matrix metalloproteases (MMPs) and their tissue inhibitors (TIMPs). Interestingly, transgenic mice overexpressing interleukin IL-13 show features consistent with airway remodelling.[30] The precise mechanisms leading to airway remodelling are not fully understood, but it has been hypothesised that the bronchial epithelium plays an important role in airway remodelling and inflammation in asthma by producing cytokines and interaction with inflammatory cells [31,32] and the extracellular matrix.[33]

Cytokines and chemokines that mediate inflammation are found to be present in broncho-alveolar lavage fluid in patients with asthma. These include elevated levels of Th2 type cytokines, such as interleukin (IL)-4, 5, 9 and 13. It is currently thought that the imbalance between Th1 (interferon-γ and IL-2) and Th2 (IL-4, 5, 9, 13) lymphocytes is a fundamental underlying mechanism in asthma.[28] The Th2 skewed immune response in asthma is shown in figure 1.[33] Environmental allergens are processed by antigen presenting cells (APC) and presented to T-lymphocytes. In the presence of IL-12, differentiation into Th1 memory cells occurs, whereas the presence of IL-10 and the absence of IL-12 stimulated the differentiation into Th2 lymphocytes that produce IL-4, IL-5, IL-9 and IL-13.[33] These cytokines will be discussed in detail in

this thesis and are summarised in table 1. Some of these cytokines act through the activation of transcription factors, such as nuclear factor-κB and members of the signal transduction-activated transcription factors family (STAT). These transcription factors subsequently upregulate adhesion molecules, pro-inflammatory cytokines and chemokines.
Asthma appears to be the result of allergic inflammation, in which the production of immunoglobulin E (IgE) is essential. Allergens that enter the airway are processed by dendritic cells that have the ability to present antigen to T- and B cells. In the presence of cytokines, such as IL-4 and IL-13, and costimulatory molecules, CD40-CD40L, B cells switch to the synthesis of IgE[28,34] (figure 2). Key effector cells in asthma in the airways include inflammatory cells such as mast cells, eosinophils and lymphocytes (especially CD4 positive T helper 2 lymphocytes) (table 1).

Genetics

This thesis builds on the tremendous progress in genetic research over the last decades. This starts in 1953 with the seminal paper of Watson and Crick on the structure of DNA and to the publication of the working draft of the sequence of the human genome in 2001 (table 2).[35-37] From this sequence, the total number of human genes is predicted to be between 30.000 and 40.000, much less than had been thought previously.[38] Complete knowledge of the DNA transcribed into mRNA and protein (the proteome) will be crucial to start understanding diseases that have a genetic origin.

Figure 2 IgE regulation

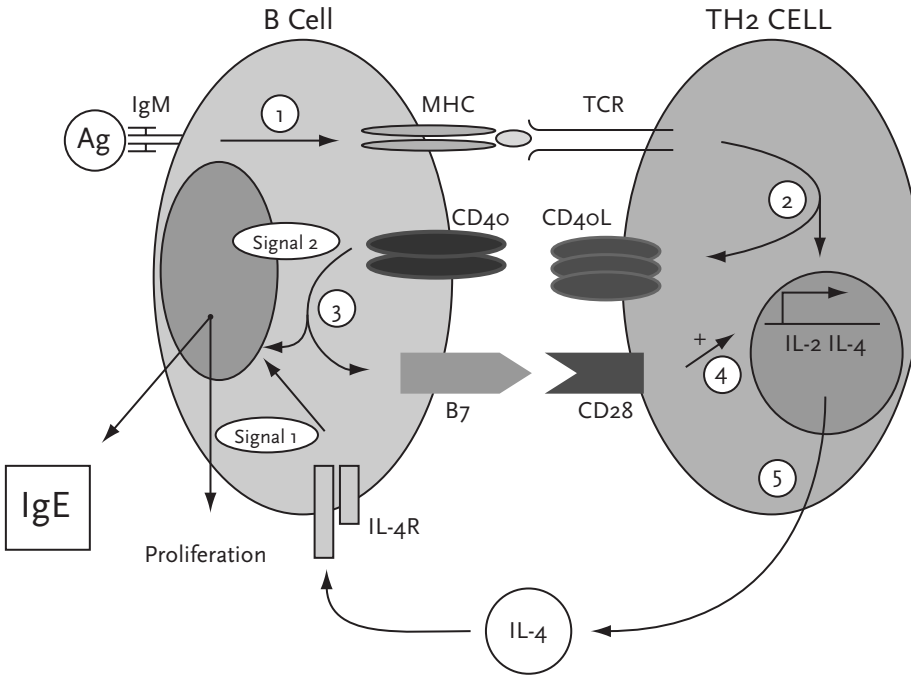


Table 1 Some cytokines relevant to development and/or progression of atopy and asthma (from Busse and Lemankse Jr (28))

Cytokine	Gene location	Primary source	Primary target	Effects or function
IFN-γ	12q14	CD4+ Th1 cells, lymphocytes, natural killer cells, some CD8+ cells	Macrophages CD4 + cells CD8 + cells Natural killer cells	Differentiation and activation Shift towards Th1 cytokine production Increased cytotoxicity of CD8+ T-cells Activation
IL-4	5q31-33	CD4+ Th2 cells	B cells Th1 cells Th2 cells CD8+ T cells Natural killer cells	Growth and activation, production IgE Inhibition Th1 cytokine production Differentiation of Th2 cells Differentiation of CD8+ T cells Inhibition of proliferation
IL-5	5q31-33	CD4+ T cells, CD8 + T cells	Eosinophils	Proliferation and activation
IL-9	5q31-33	CD4 + T cells	B cells	Enhancement of response to IL-4
IL-10	1q32-33	CD4+ Th0 cells, Th1 and Th2 cells, CD8+ T cells	Monocytes Macrophages	Differentiation to macrophages Inhibition of expression of adhesion molecules, swith Th1 to Th2 cells, inhibition IL-4 and IFN-γ production by TH2 cells
IL-12	5q31-33 (p40) 3p12-q13 (p35)	Monocytes, macrophages	Natural killer cells Th0 cells Th1 cells Th2 cells	Activation Production IL-2 Production IFN-γ and TNF-α Inhibition of production of IL-4, 5, and 10
IL-13	5q31-33	CD4+ Th2 cells	B cells Monocytes	Growth and activation, production IgE Production MHC class II molecules and integrins Inhibition of IL-2 and Il-1 and TNF

Note. IL Interleukin, TNF Tumor necrosis factor, MHC major histocompatibility complex, IFN interferon. Th T helper

Figure 2. The development of the Th_1 and Th_2 response

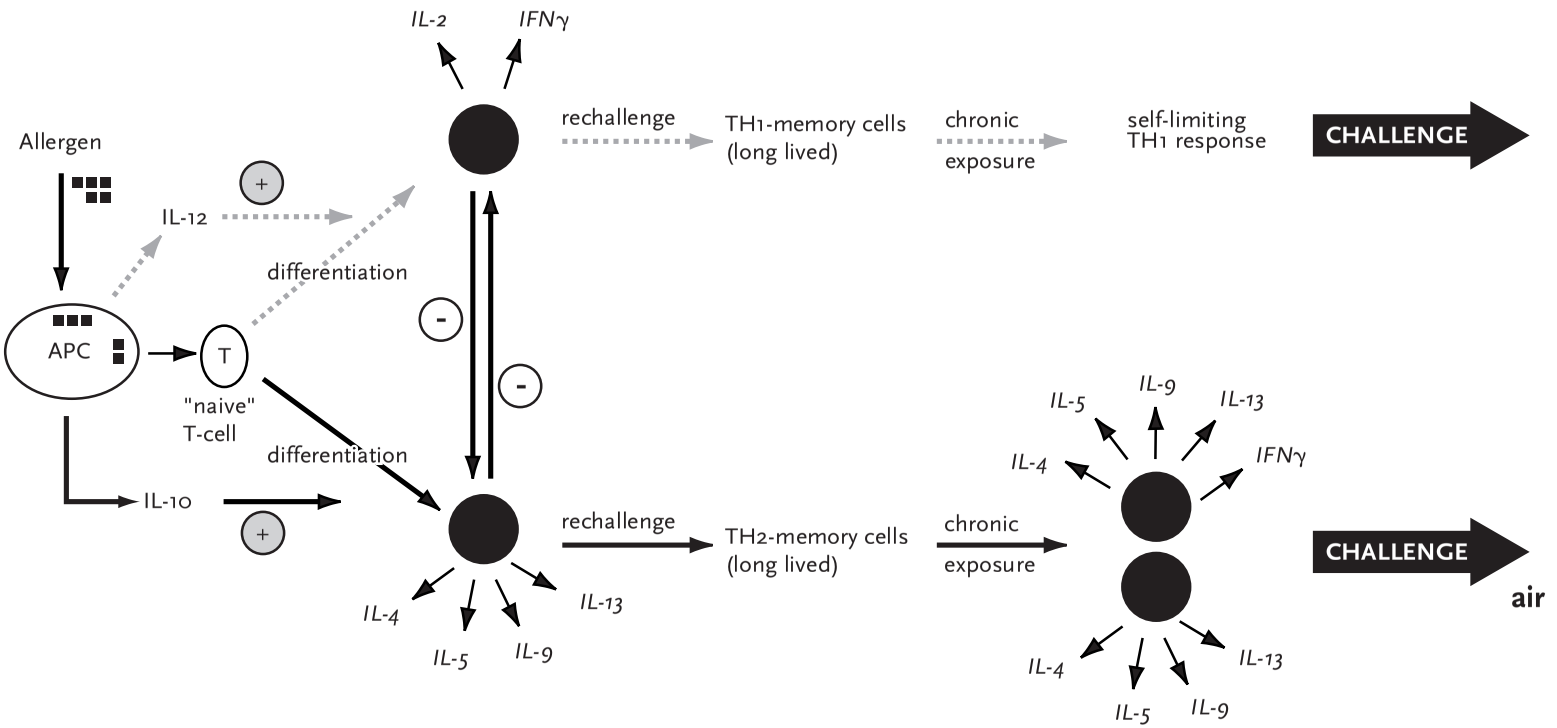


Table 2 Milestones in genetic research

Year	Milestone
1953	Watson and Crick discovered the double helical structure of DNA
1977	Two separate scientific groups proposed a way to sequence DNA
1980	A method to map the entire human genome with restriction fragment length polymorphisms (RFLP) was proposed
1985	The polymerase chain reaction (PCR) was developed
1990	Official start human genome project
1994	A first genetic linkage map of the human genome (average marker spacing 0.7 cM) is published
1995	The first sequence of a living organism, Haemophilus influenzae, is published
1997	Capillary sequencers become available
1999	The sequence of the first human chromomosome, chromosome 22, is completed
2000	The sequence of the fruit fly Drosophila melanogaster (180 Mb) is completed The sequence of chromosome 21 is completed The sequence of the first plant (Arabidopsis thaliana) is completed
2001	The working draft of the human genome is published

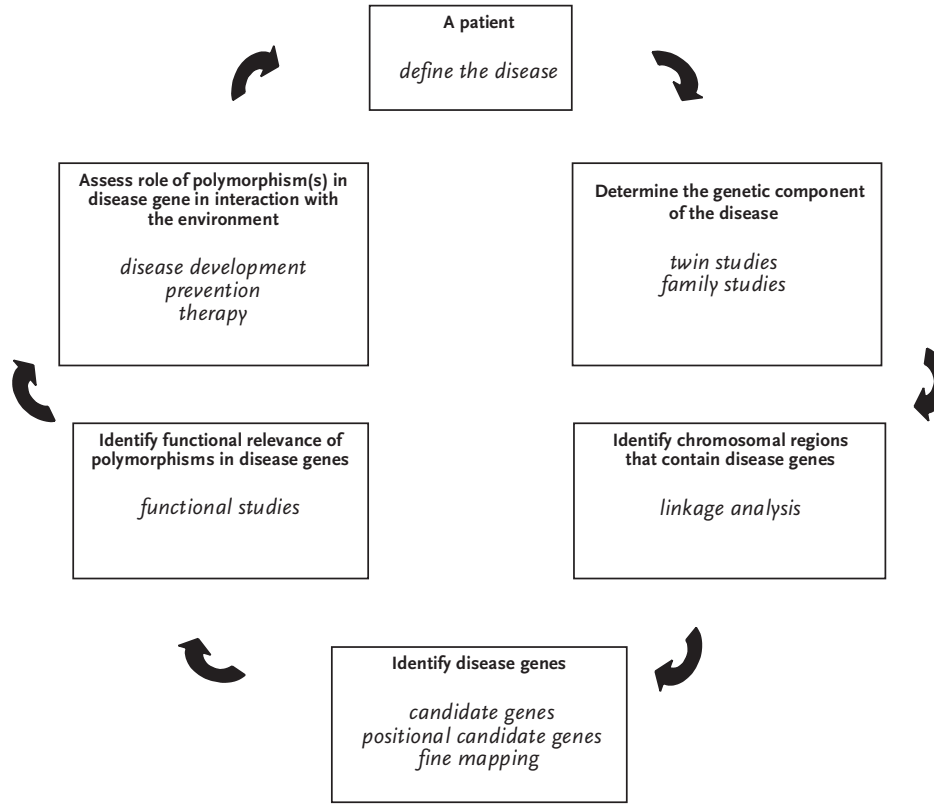
Genetic diseases, such as cystic fibrosis, are the result of the change in one gene. Chronic diseases, such as diabetes, inflammatory bowel disease and asthma, are the result of an interaction between multiple genes and environmental factors. These diseases are called genetic complex diseases.[39] Which alterations in genes confer susceptibility to these genetic complex diseases is currently not completely understood. Unravelling this may have major significance to public health with respect to early identification, prevention, and the development of personalized treatment.[39, 40] To this aim, in this thesis we will describe studies in the search for genes in asthma and atopy.

Genetic studies of complex diseases

The starting point of genetic studies of complex diseases is the patient. The key question for every medical doctor 'Why does this particular patient get this disease?' could be answered in part by pursuing the heritable origin of the disease. Genetic studies of complex diseases can be summarized as a circular process of six steps, starting and ending with the patient (figure 3).[40,41] These steps can be summarized as follows:

- Define the disease
- Determine the genetic contribution to the disease
- Identify chromosomal regions that contain disease genes
- Identify (polymorphisms in) disease genes
- Identify functional relevance of polymorphisms in disease genes
- Assess role of polymorphisms in disease genes in interaction with the environment

Figure 3 Steps in genetic research in complex diseases



Genetic research has been successful in single-gene Mendelian diseases, such as Duchenne muscular dystrophy and Huntington disease. Since the early 1990, genetic research has focussed on complex human diseases. The identification of these disease genes is a major challenge to the scientific community. Recently, it was proposed that a gene (calpaine-10) was identified for a complex disease, diabetes type II, through positional cloning.[42,43] Although further replication in other populations needs to be shown, this would provide an important proof of concept. In asthma, genetic research is still in full progress. This thesis will contain examples of studies of the genetics of asthma and atopy. It reflects the collaboration of a team of clinicians, technicians, statisticians, geneticists and molecular biologists in the Netherlands and in the United States over the past 11 years.[44]

Step 1. Define the disease

A first step is to define the trait under study. In genetics, choosing the right definition (the disease phenotype) is especially important. If this definition reflects gene function best, the chances of success to find one or more specific genes may increase. Several guidelines for defining a disease have been suggested previously by Lander and Kruglyak.[39] Issues in defining asthma and the application of guidelines to define asthma in genetic studies are discussed in chapter 2.

Step 2. Determine the genetic contribution to the disease

Involvement of genetic factors is suggested from familial aggregation of the disease. In 1650, Sennertus observed that his wife, three of her brothers and sisters and her niece all had asthma (cited in Wiener).[45] Familial aggregation of a trait can be examined using the comparison of correlations between family members. For example, total serum IgE levels was not correlated between unrelated spouses (r=-0.06), but showed significant correlations between parents and offspring in our family study (r=0.24).[46] In addition, we also observed familial clustering of skin test positivity and the presence of specific IgE to common aeroallergens. Finally, the prevalence of a disease in children with a family history of the disease can be compared to the population prevalence. In a previous study of our first 92 families, a higher prevalence of asthma was observed in first degree offspring of asthma patients (26 %) than the population prevalence of asthma (estimated 8%).[17] This indicated familial clustering of asthma.

Thus, both asthma and atopy show familial aggregation. In this thesis, family studies are reviewed in the first section of chapter 1. Important questions that are answered in that chapter are:
– Are there, according to the published literature, major genes for asthma and atopy?
– What are the number of genes involved and do we know the mode of inheritance?

Twin studies can dissect familial and environmental contribution to disease. Twin studies in asthma are discussed in chapter 3. In this short review, we asked two questions:
– What is the heritability in liability to asthma?
– What is the nature of the environmental contribution in asthma based on twin studies?

Familial aggregation of atopy is further investigated in chapter 4. In this study, we assessed the possibility of a parent of origin effect in atopy and investigated the role of environmental factors in these families as indicated by the sibling effect.

Step 3. Identify chromosomal regions that contain disease genes

The classic method to identify chromosomal regions that contain disease genes is through linkage analysis. In part 3, the results genome-wide linkage analysis of characteristics of asthma and atopy are shown. The research questions for that part are:

– which chromosomal regions may contain susceptibility genes for regulation of total serum IgE (chapter 5)
– which chromosomal regions may contain susceptibility genes for serum specific IgE, allergy skin tests and number of blood eosinophils? (chapter 6)
– which chromosomal region on chromosome 2 may contain an atopy susceptibility gene? (chapter 7)

Step 4. Identify disease genes

There are two methods to identify a disease gene. First, fine mapping of linked chromosomal regions may eventually lead to identification of disease genes. This strenuous part of genetic studies of asthma and atopy is in full progress. No successful fine mapping attempts have been published in the literature so far. However, new statistical methods based on linkage disequilibrium have become available and this may certainly facilitate gene-finding studies.[47-50] A second method is through candidate gene analysis. Candidate genes can be chosen based on understanding of the disease and / or based on the localisation on the human genome (positional candidate). Analyses of five proposed candidate genes are included in this thesis, namely CTLA-4 and CD28 (chapter 7), CD14 (chapter 8), IL-13 (chapter 9), and IL-4R (chapter 10).

Step 5. Identify functional relevance of polymorphisms in disease genes

Association per se does not fully prove the role of a gene variant in disease. It has to be complemented with functional evidence. The functional role of a gene can be assessed in various ways: an animal model (a knockout or transgenic mouse); an in vitro model (e.g. a cell system, or a reporter system to study promoter function), ex-vivo or in vivo. This thesis contains a study assessing the functional role of different promoter polymorphisms in the gene encoding the $ß_2$-adrenoceptor. Functionality of these variants was assessed by studying $ß_2$-adrenoceptor density and function in peripheral blood mononuclear cells of asthma patients (chapter 11).

Step 6. Assess role of polymorphisms in disease genes in interaction with the environment

In genetically complex diseases, genes interact with environment in the development of the phenotype. Once relevant disease genes are known, the interaction with the environment may be investigated. Next to causative factors, the interaction of the genetic background with medication may be assessed (pharmacogenetics).

Finally, a summary of the findings of this thesis is given, together with ideas on future studies.

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Chapter 1 | The genetics of asthma

(Asthma, 4th edition. London: Arnold, 2000: 146-174)

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1. Introduction

Asthma and allergies have long been recognized to have a familial basis. In a paper published in 1916, Cooke and Vanderveer studied family histories of 504 patients with allergy and concluded "that inheritance is a definite factor in human sensitisation".[1] However, the exact mechanisms that underlie this familial basis remained unknown. This situation has changed in the last two decades since new tools in molecular biology and genetic epidemiology have become available to facilitate genetic studies. The cystic fibrosis gene, discovered in 1989, follows Mendel's laws for single gene transmission. Mendelian genes show recessive or dominant patterns of inheritance in families. A current challenge is the genetic dissection of traits and diseases that do not show these Mendelian patterns. Such traits and diseases are called genetic complex diseases, which are influenced both by genes and environmental factors.[2] Examples of genetic complex diseases are multiple sclerosis, diabetes and asthma.

In general, genetic complex diseases show no simple relation of genotype and phenotype. This may be due to different genes causing the same phenotype (genetic heterogeneity) or the same genotype resulting in different phenotypes (pleiotropy). Some individuals with the mutated gene may not express the phenotype (incomplete penetrance), whereas others without the gene do show the specific phenotype (phenocopy). Furthermore, it is likely that in asthma some traits may require the presence of mutations in different genes at the same time (polygenic inheritance). These genes in turn can have different gene-gene and gene-environment interactions. Thus, mutated genes in genetic complex diseases can be regarded as risk factors for a disease comparable with other generally recognized risk factors for the development of diseases. This can be illustrated with the example of airway responsiveness. Hypothetically, genetic regulation of airway responsiveness may be influenced by two major genes. Different variants of these genes may lead to susceptibility for airway hyperresponsiveness. Environmental factors, such as smoking and allergen exposure may act as exogenous factors, resulting in airways hyperresponsiveness in susceptible individuals by different gene-environmental interactions.
The purpose of this chapter is to review the definition of asthma in genetic studies, the genetic basis of asthma and the current evidence on the localization of asthma susceptibility genes. A glossary of some genetic terms is listed in table 1.

Table 1. Explanations of genetic terms

Affected relative pair:	A set of individuals related by blood, each of whom is affected with the trait in question. The most common types of affected relative pairs include affected sibling pairs, affected cousins and affected avuncular pairs.
Affected sibling pair:	See: affected relative pair.
Allele:	Alternative variant of a gene or marker due to changes at the DNA level.
Ascertainment:	The selection of individuals for inclusion in a genetic study.
Autosome:	In humans any chromosome other than the sex chromosomes.
Candidate gene:	A gene that has been implicated in causing or contributing to the development of a particular disease.
CentiMorgan:	A measure of genetic distance, equivalent to 1% recombination.
Chromosome:	Macromolecular complex of DNA and protein. Humans have 46 chromosomes (23 pairs).
Codon:	A triplet of three bases in a DNA and RNA molecule, specifying a single amino acid.
Concordant:	A pair of relatives, mostly twins, in which both members exhibit the same phenotype or trait.
Complex trait:	A trait which has a genetic component, that is not inherited in a strictly Mendelian fashion (dominant, recessive or sex-linked).
Crossing-over:	Reciprocal breaking and rejoining of homologous chromosomes in meiosis that results in exchange of chromosomal segments.
Discordant:	A pair of relatives, mostly twins, in which both members exhibit different phenotypes or traits.
DNA:	Desoxyribonucleic acid, the molecule that encodes the genetic information in virtually all organisms.
DNA marker:	A cloned chromosomal locus with allelic variation that can be followed directly by a DNA based assay such as polymerase chain reaction.
Epistasis:	Two or more genes interacting with each other in a multiplicative fashion
Exon:	The portion of the genome that is expressed as processed mRNA.
Gamete:	Any mature germ cell.
Gene:	An individual unit of heredity. It is a specific instruction that directs the synthesis of a RNA product.
Genome:	The sum of all genetic information of an organism.
Genotype:	The observed alleles at a genetic locus for an individual.
Haploid:	The chromosome number of a normal gamete. In a gamete, only one of the two chromosomes of a chromosome pair is present.
Haplotype:	The linear, ordered arrangement of alleles on a chromosome.
Heterozygote:	A diploid organism with two distinguishable alleles at a particular locus.
Homozygote:	A diploid organism with two identical alleles at a particular locus.
Identity-by-descent:	Two alleles are identical by descent when it can be determined that they have been inherited from a common ancestor.
Imprinting:	A phenomenon in which the phenotype depends on which parent passed the disease gene.
Intron:	The non-coding regions of genes. The introns are spliced out of the mRNA following transcription.
Linkage:	Co-inheritance of two or more loci because of close proximity on the same chromosome, so that after meiosis they remain associated more often than the 50 % expected for unlinked loci.
Linkage disequilibrium	The preferential association of a particular allele, for example, a mutant allele for a disease with a specific allele at a nearby locus.
LOD score:	A statistical method that tests whether a set of linkage data indicates two loci are unlinked or linked. The LOD score is the base 10 logarithm of the odds favouring linkage.
Mapping:	The process of determining the position of a locus on the chromosome relative to other loci.
Marker:	See: DNA marker.
Meiosis:	The specialized form of a cell division that creates germ cells
Microsatellite:	A class of DNA polymorphisms arising from a short base-pair sequence that is tandemly repeated a variable number of times; microsatellites are used as genetic markers in linkage analysis.

Explanations of genetic terms (continued)

mRNA:	Messenger RNA, a type of RNA molecule that carries the information copied from a gene and serves as a template for the production of proteins.
Multifactorial:	A trait is considered to be multifactorial in origin when two or more genes, together with an environmental effect, work together to lead to a phenotype.
Mutation:	A change, deletion, or rearrangement of the DNA sequence.
Nucleotide:	The building block of RNA and DNA.
Oligogenic:	A few genes work together to produce the phenotype. Contrasted to polygenic, which implies that many genes are involved.
PCR:	Polymerase chain reaction, a technique for amplifying short stretches of DNA.
Penetrance:	The probability of expressing a phenotype given a genotype.
Phenocopy:	A trait which appears to be identical to a genetic trait, but which is caused by non-genetic factors.
Phenotype:	The observed manifestation of a genotype.
Polymorphism:	Loci at which there are two or more alleles that are each present at a frequency of at least 1% in the population.
Power:	The probability of correctly rejecting the null hypothesis.
Proband:	An individual, through which a family is ascertained for a genetic study, mostly an affected individual.
Recombination:	The formation of a new combination of genes during meiosis.
Restriction enzymes:	A group of enzymes isolated from bacteria that cut DNA molecules at specific sites characterized by specific nucleotide sequences.
RNA:	Ribonucleic acid, a ribonucleotide polymer into which DNA is transcribed.
Segregation analysis:	A method of genetic analysis that tests whether an observed pattern of phenotypes in families is compatible with an explicit model of inheritance.
Sequencing:	The process of determining the order of nucleotides in a nucleic acid or amino acids in a protein.

2. The definition of asthma in genetic studies

Asthma is a respiratory disease characterised by variable airway obstruction, airway inflammation and airway hyperresponsiveness. A major issue in genetic studies is how to define the asthma phenotype.[3] Ideally, this definition would separate 'true' asthma from other lung diseases such as chronic obstructive pulmonary disease (COPD) or healthy status. An accurate definition of asthma in genetic studies is important, as misclassification of individuals reduces the power of genetic studies to a great extent.

In defining the asthma phenotype for genetic studies, one has to recognize the marked clinical heterogeneity of the disease, with regard to its age of onset and variations in symptoms over time, its severity and the association of asthma and atopy. Furthermore, an overlap may exist between asthma and COPD. There are some clear differences between asthma and COPD, such as age of onset (asthma mainly in childhood and adolescence, COPD in older age). An example of a similarity is AH, a central feature of asthma, which can be detected in the majority of patients with COPD. Furthermore, both diseases are characterised by airway obstruction. Airway obstruction is reversible spontaneously or after the use of $ß_2$-agonists in

most patients with asthma, but not all. In contrast, airway obstruction is not reversible in most patients with COPD. However, in a 25 years follow-up study of adult asthmatic patients about 30% of these patients developed irreversible airway obstruction.[4] These patients do have asthma with irreversible airways obstruction, but without any clinical history one could easily diagnose them as patients with COPD. This example illustrates that the classification of patients with obstructive airways disease is not always easily made.

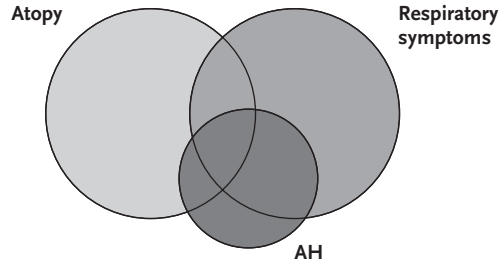
Possible approaches in defining the asthma phenotype

In genetic studies the asthma phenotype can be studied by a questionnaire that assesses self-reported wheeze or asthma, or a doctor's diagnosis of asthma. A clear advantage is that this is an easy and feasible approach which can be used in large scale studies. However, a disadvantage is that self-reported wheeze has a high population frequency and may overestimate the asthma prevalence, as some children wheeze during the course of a viral upper airway infection, but do not develop asthma.[5] Furthermore, wheeze is also present in a considerable proportion of patients with COPD. The use of a doctor's diagnosis of asthma or the use of asthma medication for classifying asthmatics has been criticized, because some evidence suggests that doctors are more likely to diagnose asthma in females, non-smokers and children who have a positive family history of allergy.[6] Furthermore, one may misclassify individuals with mild, intermittent disease because they do not attend a doctor. Thus, questionnaire-based approaches appear to have their limitations. Therefore, current studies are now directed at measuring clinical and objective characteristics, which constitute a marker of, or are associated with asthma. Examples of these traits are airway hyperresponsiveness, reversibility of airway obstruction after inhaling a ß-agonist, and markers of atopy such as total serum IgE or allergen-specific IgE levels, the number of blood eosinophils in peripheral blood and skin prick tests for common aeroallergens.

Airway hyperresponsiveness (AH) to inhaled bronchoconstrictor agents is a central feature in asthma, which can be detected in virtually all symptomatic patients. Longitudinal studies have indicated that AH is a risk factor for the development of asthma and respiratory symptoms. It can be studied by inhalation provocation either using a direct stimulus (histamine or methacholine) or indirect stimulus (adenosine-5-monophosphate, cold air, exercise or hypertonic saline).

Reversibility of airway obstruction after inhalation of a $ß_2$-agonist is often taken as a surrogate marker for AH in patients who cannot perform this test due to low lung function. However, a recent study showed that these two phenotypes are not interchangeable in the general population.[7] Variability of airway obstruction can also be assessed with serial peak expiratory flow (PEF) measurements. International guidelines have advised to use increased variability of PEF over the day as a diagnostic tool for asthma and to use it in clinical management for adjusting therapy. Although AH and variability of PEF are correlated, their different associations to allergy markers in the general popu-

Figure 1 Interrelation of airway hyperresponsiveness, atopy and respiratory symptoms



Venn diagram showing the interrelation of airway hyperresponsiveness (AH), atopy and respiratory symptoms in asthma, such as wheeze, dyspnea, cough and nocturnal asthma. This figure shows the interrelationship of these three phenotypes, which are often found together in individuals with asthma. However, these phenotypes can occur separately in individuals who do not have asthma

lation may indicate that they cannot be used interchangeably.[8] Furthermore, the genetic component of peak flow variability and reversibility has not been formally studied, and the value of this phenotype remains to be established.

Asthma has a close relation with atopy, especially in children and adolescents. Atopy can be defined as a prolonged increased production of IgE as a reaction on exposure to common antigens. Its clinical expression includes asthma, allergic rhinitis (hay fever) and atopic dermatitis (eczema). Atopy is reflected in elevated levels of serum total IgE, allergen-specific IgE levels and positive skin test to common allergens. Atopy is often accompanied by raised numbers of eosinophils in peripheral blood. The phenotypes of asthma and atopy are often interrelated (figure 1).[9,10] Therefore, in this chapter attention will be given to the complex asthma phenotype, as well as the distinct intermediate phenotypes of asthma, such as AH, reversibility, serum total IgE, allergen specific IgE, positive skin-prick tests and the total number of eosinophils in peripheral blood.

3. Asthma as a genetic disease

Asthma clusters in families. The risk that a first-degree family member of a patient with asthma will develop asthma has been calculated to be less than two to almost six times higher than the risk for individuals in the general population.[11,12,13] Both shared genes and shared environment could account for such an excess risk. Two approaches, twin studies and segregation analyses, can separate the relative contribution of genes and environment to a certain trait and are discussed below.

Twin studies

The main goal in the study of twins is to estimate the genetic and environmental contribution to a specific trait or disease. Similarities or differences are compared in monozygotic (MZ) and dizygotic (DZ) twins. Since MZ twins share 100 % of their genetic information and DZ twins 50 %, higher similarity in MZ co-twins is explained by their greater genetic similarity. The main assumptions of twin studies are: that the environment for both MZ and DZ twins is similar; that they are representative of the general population; and, in questionnaire-based studies, that self reported zygosity is correct.

The first large twin study published on asthma was a Swedish population-based study of 6996 twin pairs.[14] In this study MZ concordance for self-reported asthma was 19.0% and DZ concordance was 4.8%. This indicates that both genetic and environmental factors are important in asthma. The genetic influence is illustrated by the higher concordance in MZ twins compared to DZ twins. In contrast, environmental influences are evidenced by the finding that in genetic similar MZ twins, sometimes one of a twin pair has asthma and the other not. Since then, several other twin studies in different populations have confirmed this finding (table 2).[15-20] These twin studies provide strong evidence for the hereditary basis of asthma. Furthermore, from these twin studies one can conclude that both airway hyperresponsiveness and serum total IgE are under significant genetic control. Data of twin studies on the genetic regulation of allergen specific IgE and skin test sensitivity are, at the moment, scanty. The currently available evidence suggests that whereas the ability to produce IgE is regulated genetically, the specificity of the IgE response is governed mainly by environment (table 2).[15,21,22]

Segregation analysis

Segregation analysis tests the hypothesis that the aggregation of a trait in families is the result of the action of a major gene. It does not include molecular biological techniques or DNA analyses. This analysis compares the number of individuals with a certain trait under study in a family with the expected numbers using different genetic models of inheritance. Examples of these models are models with a Mendelian component (a dominant gene model, a recessive gene model), or non-Mendelian models such as a polygenic model (multiple genes with small effect), a mixed model (a single major gene on a polygenic background) or a non-genetic, environmental model (no evidence for genetic factors). The result of segregation analysis is the genetic model with the highest likelihood, i.e. the model that gives the best description of the segregation of the trait under study in the family data. From this model, one can estimate the mode of inheritance and parameters such as the penetrance, the heritability and allele frequencies.[23]

Segregation of the asthma phenotype has been studied in several large, questionnaire-based studies (table 3). The self-reported family history of 13 963 asthma patients participating in the European Community Respiratory Health Survey was analyzed with a complex segregation analysis. This study reveals further support for genetic regulation of asthma and provides evidence for a two-allele gene with codominant inheritance.[24] Four other

Table 2 Twin studies of asthma and asthma-associated phenotypes

Phenotype	First author, year of publication	Population	Number of twin pairs	MZ correlation+	MZ concordance#	DZ correlation+	DZ concordance#	Definition of phenotype / Comments
Asthma	Edfors-Lubs, 1971	Swedish	6996		0.19~		0.05~*	Questionnaire / population based study.
	Hopp, 1984	US	107		0.50~		0.33~	"History of asthma" by questionnaire.
	Duffy, 1990	Australian	3808	0.65		0.24*		Questionnaire.
	Nieminen, 1991	Finnish	13.888	0.43		0.25*		Hospitalization, medication or cause of death / population based study.
	Sarafino, 1995	US	94		0.59		0.24*	Questionnaire.
	Lichtenstein, 1997	Swedish	434 ♂ 456 ♀		0.62 ♂ 0.41 ♀		0.26 ♂* 0.18 ♀*	Questionnaire (ever wheezing with shortness of breath, wheezing without a cold, or parental reported asthma) / twins aged 7-9 years.
	Harris, 1997	Norwegian	2559		0.45		0.12*	Questionnaire / population based study of twins aged 18 - 25 years.
	Laitinen, 1998	Finnish	1713		0.42		0.17*	Questionnaire / population based study of twins aged 16 years.
AH	Hopp, 1984	US	107	0.67		0.34*		AH to methacholine.
Total IgE	Hopp, 1984	US	107	0.82		0.52*		
	Hanson, 1991	US apart	70	0.64		0.49*		Twins reared apart.
		US together	61	0.42		0.26		Twins reared together.
		Finnish	158	0.56		0.37*		
Specific IgE	Wütrich, 1981	German	50		0.60		0.23	At least one allergen RAST positive.
	Hanson, 1990	US apart	26		0.50		0	Specific IgE antibodies for Ambrosia artemisiifolia,
		US together	14		0.50		0.33	P. pratense and Alternaria teni were measured by RAST / apart: reared apart; together: reared together.
Skin test	Hopp, 1984	US	107	0.82		0.46*		Sum of positive intracutaneous (skin) tests.
	Hanson, 1990	US apart	39		0.55		0.50	≥1 Intracutaneous (skin) test with wheal size > 5mm
		US together	41		0.70		0.28	

* Statistically significant differences between monozygous (MZ) and dizygous (DZ) pairs. + Correlation: intrapair correlation.
# Concordance: probandwise concordance. ~ pairwise concordance. AH: airway hyperresponsiveness. Rast: radio allergo sorbent test

Table 3 Segregation analyses of asthma and asthma-associated phenotypes

Phenotype	First author, year	Number of families	Genetic model	Definition of phenotype / Comments
Asthma	Lawrence, 1994	131	Common genes of small effect	Questionnaire/ population based sample
	Holberg, 1996	906	Polygenic or oligogenic model, not a single two-allele gene	Questionnaire, physician diagnosed asthma
	ECRHSG, 1997	13.963	Two-allele gene with co-dominant inheritance could not be rejected	Questionnaire, family history of asthma was reported by proband
	Jenkins, 1997	7.394	Oligogenic model	Questionnaire/ population of school children
	Chen, 1998	309	Single locus explains a portion of wheeze that is related to respiratory allergy. Also contribution of environmental factors and/or polygenes	Questionnaire defined self-reported wheeze
AH	Townley, 1986	83	Environmental hypothesis rejected, no single autosomal locus	AH to methacholine, families with and without asthma
	Longo, 1987	40	Autosomal dominant pattern of inheritance	AH to carbachol, no formal segregation analysis performed
	Lawrence, 1994	131	Common dominant genes of small effect	AH to histamine, random population sample
Total IgE	Gerrard, 1978	173	Dominant model, dominant allele suppresses high levels of IgE	In Caucasian Americans
	Meyers, 1982	23	Mendelian co-dominant model	In US-Amish population not selected for allergy
	Hasstedt, 1983	5	No major gene, polygenic inheritance	Families selected through breast cancer probands
	Meyers, 1987	42	Mixed model with recessive inheritance of high IgE levels	In families not selected for allergic disease
	Martinez, 1994	291	Co-dominant inheritance of a major gene for high IgE levels	In hispanic and non-hispanic families
	Lawrence, 1994	131	Polygenic model	Random population sample
	Xu, 1995	92	Two locus recessive model with epistasis	Families ascertained through a proband with asthma
	Dizier, 1995	234	Recessive major gene controlling high IgE levels	Independent from specific response to allergens

ECRHS: European Community Respiratory Health Survey Group. AH: airway hyperresponsiveness

studies, each with less participants, have also shown the familial aggregation of asthma and wheeze. However, the segregation of asthma in these families was consistent with the action of multiple genes with a small effect.[25-28]

Few family studies on AH have been published. Longo *et al*. studied AH to carbachol in nonasthmatic parents of patients with asthma and a sample of healthy controls. Ten percent of the normal population showed AH, whereas 50% of the nonasthmatic parents of asthmatic children had AH. These different distributions indicate a familial clustering of AH.[29] Complex segregation analysis of AH was performed by Townley *et al*. in 83 families from the USA and by Lawrence *et al*. in 131 randomly selected families from the United Kingdom (table 3). These analyses illustrate the genetic contribution to AH, but no evidence for a single major gene for AH was found.[25,30,]

Segregation of serum total IgE has been studied most extensively (table 3). Firstly, segregation analyses of serum total IgE confirmed the results of twin studies indicating major genetic regulation of serum total IgE levels. Secondly, the mode of inheritance was assessed in several studies. These studies provide evidence for different genetic models. Using a single locus approach, best fitting models were models for a major Mendelian gene, either co-dominant,[31,32] recessive,[33] mixed model of recessive inheritance,[34] dominant[35] or, in two other studies, for polygenic inheritance.[25,36] Dizier *et al*. studied serum total IgE levels in 234 Australian nuclear families. Evidence for recessive inheritance of serum total IgE levels and significant residual familial correlations were found. However, these correlations were no longer significant when the presence of the specific immune response was accounted for in the analysis. This study suggested that regulation of serum total IgE is independent from the regulation of allergen specific IgE.[33] Xu *et al*. were the first to perform a two locus approach to the fit the serum total IgE data in 92 Dutch families ascertained through a proband with asthma. This resulted in a significantly better fit of the data than a one-locus model, thereby providing evidence for two unlinked loci regulating serum total IgE in these families. The first locus alone explained 50.6% of the variance of the level of serum total IgE, the second 19.0%. Considered jointly, the two loci account for 78.4% of the variability of serum total IgE levels in serum.[37]

To date, there are no data on the segregation of other asthma-associated phenotypes in families.

In summary, segregation analyses of asthma and airway hyperresponsiveness confirm their genetic background, but are not conclusive on the mode of inheritance and the number of genes involved. Evidence for a major gene regulating serum total IgE was provided by studies in different countries, and evidence for different genetic models was obtained. Several explanations may be given for these contradictory results. A first explanation may be the definition of the phenotype. The definition of asthma and BHR varies between studies (table 3). A second explanation may be the ascertainment of families for segregation studies. In families ascertained for asthma, estimates on allele fre-

quencies of alleles regulating serum total IgE may be higher than in families sampled randomly from the general population. A final explanation may be genetic heterogeneity. This means that in different populations, different genes act in the regulation of these phenotypes. To date, this cannot be investigated since the exact localisations of these genes are still unknown.

4. Finding genes for asthma

The human genome

The haploid human genome consists of approximately 3×10^9 basepairs. Generally, genetic distances are expressed in centiMorgans (cM), one cM corresponding to 1% recombination and approximately 1 000 000 base pairs on a physical map. One percent recombination means that a crossing-over between two loci occurs every one in hundred meioses. Roughly, every one cM contains 50 genes. DNA is organised into 22 pairs of autosomes and two sex-specific chromosomes. Each chromosome comprises two arms, the short arm denoted as 'p' and the long arm as 'q'. Every region of a chromosome has been assigned a number, for example for chromosome 1, the regions are called 1q21, 1q22, 1q23, etc.

The total number of genes is estimated as many as 65.000 to 80.000.[38] At a given place in the genome, called a locus, different variants are called alleles. Many genes have a number of alleles in the population and are therefore said to be polymorphic. The majority of the DNA is not coding for any biological product. In these non-coding regions, polymorphisms can also be detected. These polymorphisms are typically used as markers for genetic studies. Thus, in this respect the words 'markers' and 'polymorphisms' are used interchangeably. In general, two different strategies have been used to identify susceptibility genes for asthma and atopy. The first strategy is positional cloning, the second is the candidate gene approach.

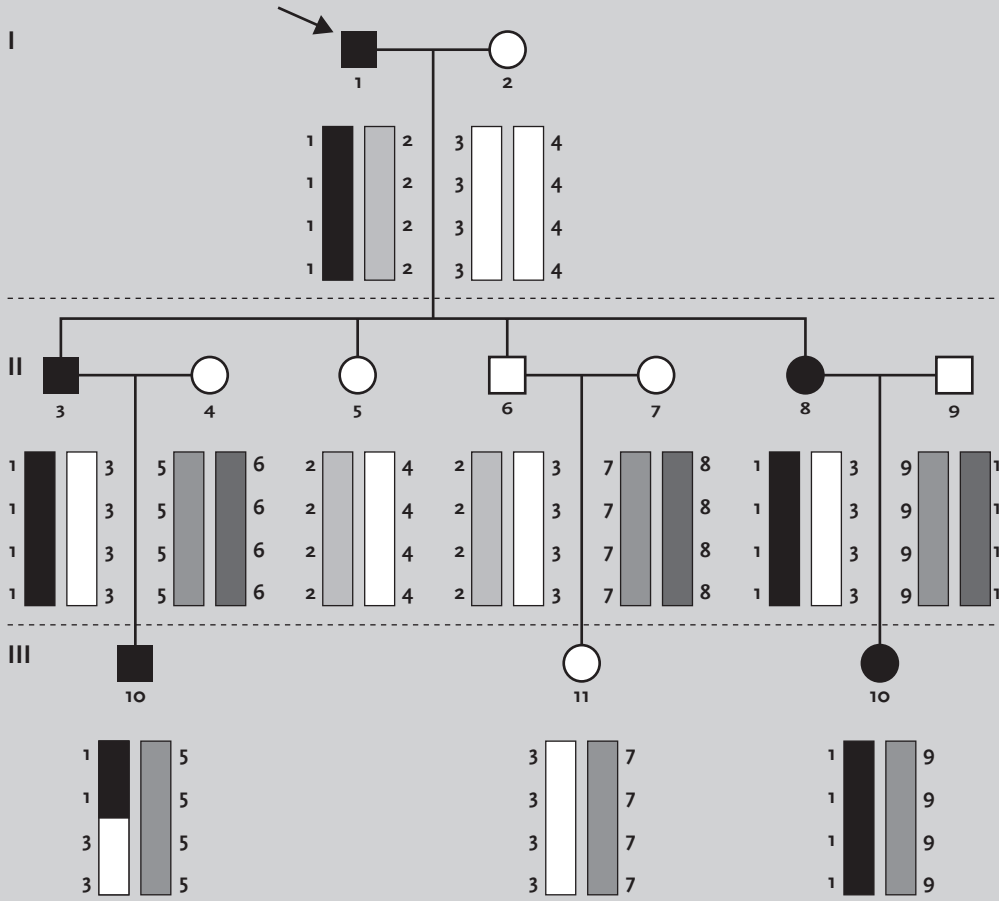
Positional cloning

The first step is to identify chromosomal regions of interest that may harbour disease genes by linkage analysis. The second step in positional cloning is to narrow down the region of interest as far as possible. Finally, in the last step the genes in this specified region are checked for mutations associated with the disease.

The principles of linkage analysis are shown in figure 2. Finding linkage is to determine a chromosomal region (sometimes millions of base pairs in length) which cosegregates with a certain trait within families. The likelihood that a trait cosegregates with a marker is expressed as a LOD score. The LOD score is the logarithm of the likelihood ratio of linkage versus no linkage. To study linkage using a LOD score approach, a model has to be specified for different genetic parameters such as mode of inheritance, penetrance, and allele frequencies. These parameters can sometimes be estimated from segregation analyses. However, given that in most studies these parameters are unknown, most investigators prefer non-parametric approaches.

A non-parametric approach is a method that does not need specification of a genetic model. Examples are affected sibling pair analysis and affected relative pair analysis. These non-parametric approaches test whether the inheritance of a chromosomal region is not consistent with random segregation. If this is the case, affected relatives inherit identical copies of alleles in this region more often than would be expected by chance.[2] The observed and expected distributions of alleles can be tested with a χ^2-test.

Figure 2 Pedigree of a family with asthma



A fictive family with asthma. Affected family members are shown as black boxes (males) or black circles (females); unaffected individuals are represented by the open boxes (males) and open circles (females). The proband is indicated by an arrow (individual 1). This family consists of three generations, as indicated by the roman capitals I, II, and III. The grandfather (individual 1), the oldest son (individual 3) and the youngest daughter (individual 8) and two grandchildren (individuals 10 and 12) are affected. On a chromosome, four subsequent markers are typed. The different alleles are coded by different numbers. In this example the trait asthma cosegregates with the haplotype of four markers with allele 1 (black chromosome). It is said that this trait is linked to the marker. Furthermore, a crossing over is observed in individual 10. From the fact, that this person is still affected, one can deduce that the gene causing this trait is located upstream of the third marker.

Candidate gene approach

Candidate genes can be detected in the process of positional cloning. In addition, investigators may choose a certain, known, gene as a plausible candidate gene for asthma. In general, candidate genes are tested with the use of association analysis in which alleles of candidate genes are tested using a case-control design. The frequency of an allele in a gene or a marker is compared between affected individuals and unaffected individuals. The finding of a positive association of an allele and a trait can be interpreted in three ways:[2]

1. the allele of interest is the relevant mutation in the disease gene;
2. the allele is in linkage disequilibrium, that means it is physically very close to the disease gene;
3. the association is a result of population admixture. This occurs if a certain trait has a higher prevalence in an ethnic subgroup within a mixed population. Any allele with a higher frequency within this subgroup will show association with the trait.

A method of testing for linkage and association is the transmission disequilibrium test (TDT).[39] Alleles of heterozygote parents are divided in transmitted and non-transmitted alleles, and the preferential transmission of a certain allele to an affected child is tested.

Results of linkage studies in asthma and atopy

The most frequently studied chromosomal regions that may harbour asthma and/or atopy susceptibility genes are chromosomes 11q, 5q and 12q (table 4).

Chromosome 11q

In 1989, Cookson *et al*. were the first to report linkage of atopy on chromosome 11q.[40] In this study, atopy was defined as one of either elevated serum total IgE, raised allergen specific IgE or the presence of one or more positive skin prick tests. Seven families were studied, whereas most of the LOD score was contributed by a single family using an autosomal dominant mode of inheritance. These authors replicated this finding in other samples, one of which was an Australian sample.[41-43] In addition, in other studies from the Netherlands, Germany, Japan and Australia, evidence for linkage was found between different asthmatic and/or atopic phenotype and markers on chromosome 11q. In a Dutch sample of 26 sib-pairs linkage was found between 11q and asthma and atopy defined as the presence of two respiratory symptoms and elevated specific or serum total IgE levels.[44] In a German study linkage was found between 11q and a clinical history of atopy and an elevated serum total IgE level[45] and in a Japanese study linkage was found between 11q and severe atopy (total serum IgE >400 IU/ml; three or more positive intradermal skin tests > 9mm or three or more positive RAST scores) in four selected families.[46] In an Australian study no linkage between chromosome 11q and atopy was

Table 4 Linkage analyses of asthma and airway hyperresponsiveness

Phenotype	Chromosome, + or – result	First author, year	Number	Genetic analysis	Definition of phenotype / Comments
Asthma	5q +	Noguchi, 1997	41 s	Sib-pair	Intermittent episodes of wheeze and dyspnea.
		CSGA, 1997	79 f	Affected relative pair	Two of three symptoms (cough, wheeze, dyspnea) and AH to methacholine or reversibility / Modest evidence of linkage.
		Ober, 1998	361 n	TDT, LR test	Bronchial hyperresponsiveness to methacholine and/or symptoms of asthma.
	5q -	Kamitani, 1997	45 s	Sib-pair	Wheeze or use of asthma medication in past year/ Random population sample.
		Laitinen, 1997	157 f	Affected relative pair, association	History of asthma, wheezing by auscultation, reversibility and/or AH.
	11q +	Herwerden, 1995	123 s	Sib-pair	Episode of asthma in the past 12 months, nocturnal shortness of breath or use of asthma medication.
	11q -	Noguchi, 1997	44 s	Sib-pair	Intermittent episodes of wheeze and dyspnea.
	12q +	Barnes, 1996	29 f	Sib-, relative pair, TDT	Reported history of asthma, confirmed by a doctor diagnosis.
		CSGA, 1997	79 f	Relative pair	Two of three symptoms (cough, wheeze, dyspnea) and AH to methacholine or reversibility after bronchodilator use.
		Ober, 1998	361 n	TDT, LR test	Bronchial hyperresponsiveness to methacholine and/or symptoms of asthma.
		Wilkinson, 1998	240 f	Sib-pair	Wheeze and asthma, defined as a quantitative asthma score
		Wjst, 1999	156 s	Sib-pair	Clinical history of asthma and ≥ 3 years of recurrent wheezing
AH	5q +	Postma, 1995	35 s	Sib-pair	AH to histamine / families ascertained through a proband with asthma.
	5q –	Doull, 1996	131 f	Association	AH to histamine / random population.
		Kamitani, 1997	51 s	Sib-pair	AH to methacholine / random population.
		Mansur, 1998	181 n	Association	AH to methacholine / random population, weak association.
	11q +	Doull, 1996	131 f	Association	AH to histamine / random population.
		Herwerden, 1995	123 s	Sib-pair	AH to methacholine / linkage even in absence of atopy.
	11q -	Lympany, 1992	9 f	LOD	AH to methacholine / in children aged 2-8 years exercise challenge test.
		Amelung, 1998	83 f	Sib-pair	AH to histamine / one marker at 11q had a modest significance level.

–: negative or not confirmative results; +: positive results.
F: number of families; s: number of sibpairs; n: number of individuals.
Sib-pair: affected sibling pair analysis. Association: association analysis. TDT: Transmission disequilibrium analysis; LOD - LOD score analysis.
LR test:likelihood ratio test, a semiparametric test for linkage. AH: airway hyperresponsiveness. CSGA: Collaborative Study on the Genetics of Asthma.

found. However, AH to methacholine appeared to be linked to 11q.[47] Linkage of chromosome 11q to atopy and asthma is still controversial due to multiple failures to replicate this finding in several other populations (table 4).[48-55] In 1992, Cookson *et al*. suggested that maternal inheritance of atopy may have obscured linkage in other studies. Excess sharing of maternal, not paternal alleles on chromosome 11q was shown in atopic children.[56] Possible explanations for maternal inheritance of atopy are paternal imprinting or maternal modification of the developing immune response. A candidate gene for atopy in this chromosomal region, the ß-chain of the high affinity IgE receptor, will be discussed in the next section of this chapter.[57]

Chromosome 5q

Chromosome 5q31-q33 contains numerous candidate genes for asthma and atopy, such as a cluster of cytokine genes (interleukine-3 (IL-3), IL-4, IL-5, IL-9, IL-13, the ß-chain of IL-12) and the genes coding for the $ß_2$-adrenergic receptor, CD-14, the corticosteroid receptor and the granulocyte-macrophage-colony stimulating factor.

In 1994, linkage between total serum IgE levels and chromosome 5q was first reported in a US Amish population.[58] This finding was replicated by Meyers *et al*. in the same year in a study of Dutch families who where ascertained through a proband with asthma.[59] In 1995, Postma *et al*. showed in the latter population that AH to histamine was linked between the same region of chromosome 5q as serum total IgE (figure 3). These findings indicated that a gene governing AH is located near a gene regulating serum total IgE.[60] Studies of asthma in Japan[54], the United Kingdom[61] and the USA[62,63] also implicated chromosome 5q as a region containing one or more susceptibility genes for asthma. However, in Australian,[64] Finnish,[65] British[66] and German populations[67] and in four US families,[68] chromosome 5q did not appear to be linked to asthma or atopy.

Table 3 Linkage analysis for total IgE and airway hyperresponsiveness (AH). Results of affected sib-pair analysis of chromosome 5q in a Dutch sample.

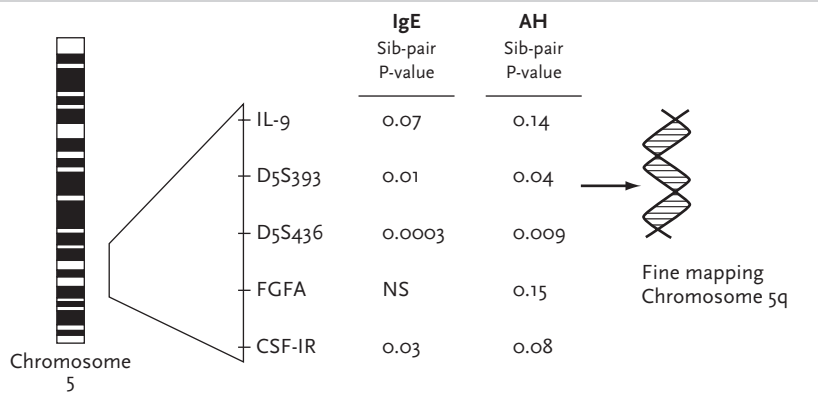


Table 5 Results of linkage analyses of atopy and total IgE

Phenotype	Chromosome, + or – result	First author, year	Number, type	Genetic analysis	Definition of phenotype / Comments
Atopy	5q +	Noguchi, 1997	71 s	Sib-pair	Total IgE >1 SD of the Japanese mean and/or elevated allergen specific IgE.
	5q -	Kamitani, 1997	103 s	Sib-pair	Skin prick test positivity to common aeroallergens.
		Laitinen, 1997	157 f	Affected relative pair, association	
	11q +	Cookson, 1989	7 f	LOD	Either of ≥1 positive skin prick test, elevated specific or total IgE.
		Cookson, 1992	70 f	Sib-pair	idem / Increased sharing of maternal, not paternal alleles.
		Young, 1992	64 f	LOD	≥1 positive skin prick test, elevated specific or total IgE.
		Collee, 1993	26 s	Sib-pair	≥ 2 respiratory symptoms and elevated total serum IgE or elevated specific IgE.
		Shirakawa,1994	4 f	LOD	Serum IgE > 400 IU/ml, ≥3 positive intradermal skin tests or ≥ 3 positive RAST scores.
		Daniels, 1996	80 f	Sib-pair	Sum of the skin-prick tests to grasses and house dust mite / Genome-wide search.
		Fölster, 1998	12 f	Affected relative pair, LOD	Clinical diagnosis of atopy and elevated total IgE levels / Families with atopic dermatitis, two- locus analysis with recessive-dominant model showed linkage in 2 of 12 families.
	11q -	Lympany, 1992	9 f	LOD	Positive skin prick test and or positive allergen specific IgE.
		Rich, 1992	3 f	Sib-pair, LOD	Either of ≥1 positive skin prick test, elevated specific or total IgE .
		Hizawa, 1992	4 f	LOD	Either of ≥1 positive skin prick test, elevated specific or total IgE, other definitions tested.
		Coleman, 1993	95 f	Sib-pair, LOD	idem / Family ascertainment through two first degree family members with atopic eczema.
		Brereton, 1994	12 f	Sib-pair, LOD	≥1 positive skin prick test.
		Martinati, 1996	45 f	Sib-pair	Either of ≥1 positive skin prick test, elevated specific or total IgE.
		Noguchi, 1997	70 s	Sib-pair	Total IgE >1 SD of the mean of the Japanese population and/or elevated allergen specific IgE.
		Amelung, 1998	83 f	Sib-pair, LOD	Elevated total IgE or number of positive skin tests.

Table 5 Results of linkage analyses of atopy and total IgE (continued)

Phenotype	Chromosome, + or – result	First author, year	Number, type	Genetic analysis	Definition of phenotype / Comments
Total IgE	5q +	Marsh, 1994	11 f	Sib pair, LOD	Eleven Amish extended families.
		Xu, 1995	92 f	Two locus LOD	First locus at 5q, second locus not mapped. (Also Meyers, 1994)
		Doull, 1996	131 f	Association	Random population sample.
		Noguchi, 1997	71 s	Sib-pair	Families ascertained through asthmatic children.
	5q -	Blumenthal,1996	4 f	Sib-pair, LOD	
		Ulbrecht, 1997	395 n	Association	Population sample.
		Mansur, 1998	181 n	Association	Population sample.
	11q +	Daniels, 1996	80 f	Sib-pair	Genome-wide search.
	11q -	Watson, 1995	131 f	LOD	Random selected families with a minimum of three children.
		Amelung, 1998	83 f	Two locus LOD	Families ascertained through a proband with asthma.
	12q +	Barnes, 1996	29 f	Sib-pair, TDT	Afro-Caribbean families ascertained through a proband with asthma.
		Barnes, 1996	24 f	Sib-pair, TDT	Amish families ascertained through one child with detectable allergen specific IgE.
		Nickel, 1997	52 n	TDT	German children ascertained from population study for high total IgE levels.

–: negative or not confirmative results; +: positive results.
f: number of families; s: number of sibpairs; n: number of individuals.
Sib-pair: affected sibling pair analysis. Association: association analysis. TDT: Transmission disequilibrium analysis; LOD - LOD score analysis.
SD: standard deviation

Chromosome 12q

Chromosome 12q is an interesting region for both asthma and atopy, because of several candidate genes, including interferon-γ (an inhibitor of IL-4 production by Th2 lymphocytes), a mast cell growth factor, and the ß subunit of nuclear factor-Y which possibly upregulates transcription of both IL-4 and the human leucocyte antigen class D-genes.

Barnes *et al*. studied individuals with a doctor diagnosed asthma and individuals with elevated total serum IgE levels in two different populations: an Afro-Caribbean population from Barbados and Caucasian Amish kindreds from Pennsylvania, USA. Evidence for linkage and association was found to this chromosomal region for both elevated total serum IgE (Barbados and Amish) and for doctor diagnosed asthma (Barbados).[69] Linkage of high serum IgE levels to 12q15-q24.1 was replicated in a German population sample of 52 children selected for high serum IgE levels.[70] Finally, evidence for linkage of asthma and 12q was shown in 240 families from the United Kingdom [71] and a study in the Hutterites in the USA.[63]

An interesting finding is that the chromosomal regions on 12q implicated in these studies are not exactly the same. Further studies are needed to fine-map this region. These studies will have to answer the question of whether one or more regions on 12q are implicated in asthma and atopy.

Other chromosomal regions of interest detected by genome-wide searches

To date, four genome-wide searches on asthma and atopy have been published. In genome-wide searches, the whole genome is scanned with markers spaced every 10 to 20 cM. The goal of a genome-wide search is to detect regions of interest for asthma and atopy using modest criteria of significance. These criteria could lead to the detection of new regions that contain susceptibility genes, as well as some regions, that could represent false-positive results. Therefore, these regions need to be followed up by additional mapping studies before a definitive conclusion can be drawn. The results from the four genome-wide searches on asthma and atopy will be discussed in this section.

In the first published genome-wide search in an Australian and British sample, evidence for linkage was found on chromosome 4 (AH), chromosome 6 (eosinophils), chromosome 7 (AH), chromosome 11 (skin tests, serum total IgE) and chromosome 16 (serum total IgE).[43]

The second genome-wide search was a US multicenter study in 140 asthma families ascertained through two or more affected siblings with asthma. Three different racial groups, namely Hispanics, Caucasians and Afro-Americans were studied.[62] An interesting result is that different regions appeared to be linked in these different racial groups. Regions of interest for asthma were chromosome 5p and 17p in African Americans; 11p and 19q in Caucasians and 2q and 21q in Hispanics.

A third genome-wide search was performed in the Hutterites. This is a religious sect that originated in Europe. In 1870, 900 members of this population moved to the USA. The current Hutterite population originates from

less than 90 ancestors, and is therefore a homogeneous population. In this study, asthma was defined as 'strict' asthma if the subjects showed AH to methacholine and reported asthma symptoms. Asthma was defined as 'loose' asthma if subjects had either AH to methacholine or reported asthma symptoms. Regions of interest for 'strict' asthma were chromosome 19q and 21q. In addition, regions of interest for 'loose' asthma were 5q and 12q. Finally, a region of interest for 'loose' asthma, not reported in other studies, was chromosome 3p. In conclusion, even in a homogeneous population such as the Hutterites, multiple susceptibility genes may influence asthma phenotypes.[63]

Finally, a fourth genome screen was performed in German families with asthma. Asthma was defined by clinical history and supported by questionnaire data of a history of at least 3 years of recurrent wheezing in children over age 3. For asthma, four possible linkages were reported at chromosome 2p, 6p, 9q and 12q. These linkage results for asthma were repeated with the study of intermediate phenotypes of asthma and atopy. For chromosome 2p, evidence for linkage was found for AR to methacholine, specific and total IgE; for chromosome 6p for total and specific IgE and eosinophils; for chromosome 9q for total and specific IgE and finally for chromosome 12q for specific IgE.[13]

Having reviewed the linkage results for asthma and atopy, some conclusions can be drawn. First, chromosome 5q, 11q and 12q are the most cited regions of interest for asthma and atopy. These findings of linkage are an important step towards the actual identification of susceptibility genes for asthma and atopy in these regions. Second, replication of linkages in other populations has proven to be difficult. Several possible reasons may explain this difficulty. One explanation may be that the definition of the phenotypes and genotypes under study are often different between studies (table 4). For example, atopy has been defined in one study as a positive history of atopic disease, in another study as a positive skin prick test and finally, in some other studies as a combination of elevated serum total IgE, allergen specific IgE or positive skin prick tests in different studies. Another explanation may be a high degree of genetic heterogeneity. This means that different genes are important in different populations; and each of these genes is sufficient to express the phenotype. In addition, several major and minor genes may interact in order to express the phenotype (oligogenic inheritance). One gene may be more prevalent in one population, whereas the second or third gene is more prevalent in other populations. Nevertheless, they provide the same phenotype. Yet another explanation may be that some of the published linkage results represent false-positive results. A final explanation is that some studies do not have a sufficient sample size to detect linkage, which may have led to false-negative results. Therefore, it is crucial that linkage results are replicated by different investigators in different populations of sufficient size. Thereafter, confirmed regions can be studied in detail and candidate genes can be detected.

5. Candidate genes for asthma

After determining linkage between asthma and a chromosomal region, the next challenge is to screen this region for candidate genes. A candidate gene for asthma has to meet four criteria:

(i) the gene product must be functionally relevant to asthma;
(ii) mutations within the gene must alter the function of the gene;
(iii) asthma needs to be linked to the chromosomal region harbouring the candidate gene and
(iv) asthma has to show association with different alleles of this candidate gene.

To date, a number of candidate genes for asthma and atopy have been studied. These include the gene encoding the $ß_2$-adrenergic receptor and genes from the cytokine cluster at chromosome 5q31-q33; the gene encoding the ß chain of the high affinity IgE receptor at chromosome 11q13, the gene encoding the interleukin 4 receptor α chain at chromosome 16p, and the major histocompability complex and the gene encoding interferon-γ at chromosome 6. Moreover, some other candidate genes for asthma and atopy will be discussed in this section.

The $ß_2$-adrenergic receptor

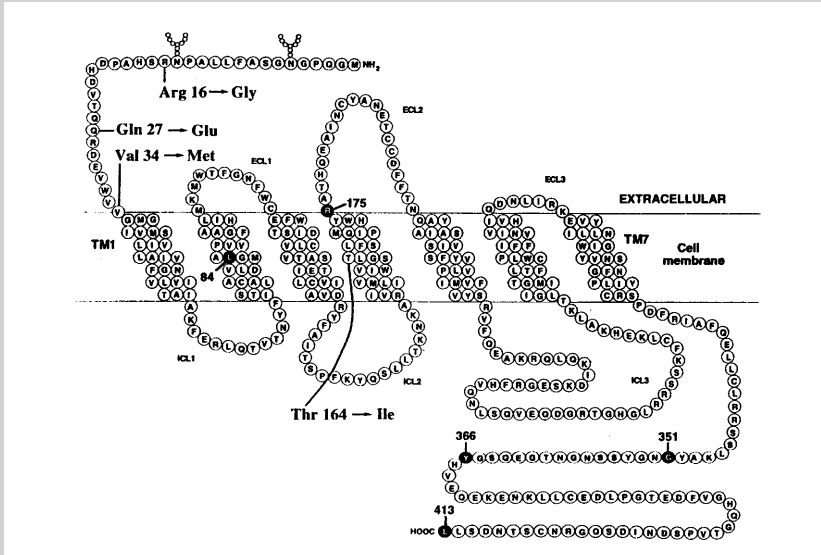
The $ß_2$-adrenergic receptor was hypothesised to play a role in the pathogenesis of asthma by Szentivanyi in 1968.[72] $ß_2$-Adrenergic receptors are localized in several human tissues and cells, including lung tissue (for instance, in airway smooth muscle and epithelium) and inflammatory cells (mast cells, macrophages, eosinophils and T-lymphocytes). $ß_2$-Adrenergic receptor function is mainly regulated by circulating epinephrine and mediates most of the effects of $ß_2$-agonists on airway function.[73]

The gene coding for the $ß_2$-adrenergic receptor is situated on chromosome 5q31. This receptor is a protein of 413 amino acids. In this gene, nine polymorphisms were identified, of which four lead to altered amino-acid sequences at positions 16, 27, 34 and 164 (figure 4). In most studies on polymorphisms of the $ß_2$-adrenergic receptor, none of these polymorphisms contributes to the risk of developing asthma. [74-76] Current evidence suggests that the polymorphisms at position 16 and 27 may play an important role in modifying the clinical severity of asthma.

Amino-acid 16 of the $ß_2$-adrenergic receptor can either be glycine (Gly) or arginine (Arg). The Gly-16 variant of the receptor might be associated with nocturnal asthma and with more severe asthma, as evidenced by the finding that patients with the Gly-16 variant were more likely to use corticosteroids and immunotherapy.[75,77] Furthermore, in a population study of children, individuals with the Gly-16 variant showed a decreased short-term bronchodilation after the use of a short-acting ß-agonist compared to individuals with the Arg-16 allele. The investigators suggest that the low response of the Gly-16 variant following ß-agonist stimulation could be a

reason for an increased use of inhaled corticosteroids.[76] The Gly-16 and Arg-16 variants of the ß$_2$-adrenergic receptor may also regulate receptor downregulation after long term exposure. Studies in airway smooth muscle cell cultures showed that long-term ß-agonists exposure downregulates the Gly 16 variant of the ß$_2$-adrenergic receptor to a greater extent than the Arg-16 variant.[78] Another study in patients with asthma showed a greater degree of agonist promoted receptor downregulation to be associated with the Gly 16 variant, not with the Arg 16 variant.[79]

Figure 4 Primary amino-acid sequence of the human ß$_2$-adrenergic receptor



Primary amino-acid sequence and proposed membrane topography of the human ß$_2$-adrenergic receptor. The darkened circles indicate codons where degenerate polymorphisms of the receptor gene were found. The four polymorphisms which result in changes in the amino-acid sequence are indicated.

The amino-acid 27 of the ß$_2$-adrenergic receptor can either be a glutamine (Gln) or a glutamate (Glu). The Gln-27 variant was associated with elevated levels of serum total IgE in a family study of 60 families with a proband with asthma. This variant was associated with more severe airway responsiveness compared to the Glu-27 variant as well.[74,80] The Glu-27 variant of the ß$_2$-adrenergic receptor showed an attenuated downregulation after the use of a long-acting ß-agonist in another study; however, this "protective" effect seemed less important than the effects of downregulation of the Gly-16 allele.[79]

In general, it is difficult to study these variants in human populations separately, since in most populations these alleles are in linkage disequilibrium. This means that the alleles at the 16 and 27 position of the ß$_2$-adrenergic

receptor are not distributed randomly in the population.
Since new polymorphisms have been recently detected in a regulatory region of the ß$_2$-adrenergic receptor, in the future more studies may be expected on the role of polymorphisms of this gene in asthma.[81]

The ß chain of the high affinity IgE receptor

The high affinity IgE receptor (FceRI) is composed of three subunits: one α, one ß and two γ-subunits. This αßγ$_2$-complex is found on the surface of mast cells, basophils, eosinophils and Langerhans cells. The binding of allergen to receptor-bound IgE on mast cells leads to activation and excretion of cytokines such as IL-4, thus upregulating IgE production by B-lymphocytes. The α-subunit is responsible for ligand binding and the γ dimer mediates for both the assembly of the receptor as well as signal transduction. The ß-subunit amplificates the signal strength mediated by the γ-subunit.[82]
Whereas the α and γ chains did not appear to be associated to asthma or atopy,[83] the ß-chain has received considerable interest.[57] The gene encoding the ß-chain is situated on chromosome 11q. As we have reported in the section on linkage, this chromosomal region was linked to asthma and/or atopy in several studies. In 1994, Shirakawa *et al*. reported that in a random British population sample an isoleucine (Ile) to leucine (Leu) change at position 181 in this protein was significantly associated with atopy if the Leu-181 variant had been inherited maternally. Of the 60 families of allergic asthmatic probands under study, this variant was detected in 10 families. In addition, at position 183 a valine (Val) to leucine (Leu) change was found.[84]
The combination of Leu-181/Leu-183 was found in 4.5 % of 1004 members of 230 two generation families in Western Australia. When inherited maternally, the Leu-181/Leu-183 variant was associated with atopy.[85] However, the Leu- 181/Leu-183 variants were not detected in other populations from Japan[86], the UK[87], Italy[53] and the Netherlands.[55]
Other polymorphisms in this gene result in two restriction sites for the restriction enzyme *Rsa* I. One of these variants was associated with atopic disease in a Japanese population[88] and atopic dermatitis in a British population[89]. However, in another Japanese study the association between these variants and atopy could not be confirmed.[90]
The most recent mutation reported is a substitution of Glu for Gly at amino acid 237 (E237G). The population frequency of this mutation is about 5% in Australian and Japanese populations. In two studies this mutation was strongly associated with asthma, and in the Australian study with AH as well.[91,86] In summary, the question remains if the Ile-181 and Ile-183 variants in the gene that codes for the ß-chain of the high affinity IgE receptor can account for the linkage reported by several groups, as it is detected in a subset of families or not detected at all in some populations. In addition, little is known on altered function of one of these variants in relation with atopy. It is therefore plausible, that other variants of this gene, such as the E237G variant, or other genes on chromosome 11q, are more important in atopy and atopic asthma.

The interleukin 4-receptor α chain

Both interleukins 4 and 13 and their receptors are candidate genes for asthma and atopy, given that IL-4 and IL-13 are central in the switch of B-cells to produce IgE and IL-4 stimulates the maturation of TH-0 to TH-2 type lymphocytes. The IL-4 receptor and the IL-13 receptor share the IL-4 receptor α-chain. The interleukin 4-receptor α gene resides at chromosome 16p. In a study from Germany, this chromosomal region was linked to markers of atopy. Only alleles inherited from the mother appeared to increase the risk on atopy in children.[92] The interleukin 4-receptor α chain has 13 known polymorphisms. Most data are available of one extracellular variant (Ile50Val), and two intracellular variants (Pro478Ser and Arg551Gln).[93] The Ile50 allele was associated with atopic asthma in one Japanese population, [94] but this could not be confirmed in another Japanese population.[95] The Arg551 allele was associated with high total serum IgE levels in a study of subjects with hyper-IgE syndrome and eczema.[96] In these studies, other polymorphisms of the IL-4 receptor α chain were not investigated. In a German population, the combination of Pro478 and the Arg551 allele was associated with lowered total serum IgE levels.[97] Arg551Gly and Pro478Ser are in linkage disequilibrium in most populations; therefore, the association of one allele with total IgE or asthma can not be studied separately. Given the multiple polymorphisms in this gene, and the contradictory association results, several groups have attempted to study the functional role of these polymorphims. First, from in vitro studies with transfected cell lines, Mitsuyashu and coworkers provided evidence that Ile50 allele, but not Arg551 allele, is involved in increased STAT-6 activation and proliferation and transcription of the IgE promoter by IL-4.[98] Second, from in-vivo immunoassays using T cells of individuals with different alleles of the Pro468Ser and the Arg551Gly, Kruse and coworkers suggested that the phosphorylation status of transcription factors IRS-1, IRS-2 and STAT6 was changed in the presence of these polymorphisms.[97] In conclusion, although the genetic associations and the available functional data indicate the importance of the IL-4R gene, more research is needed to clarify the role and the interaction of these polymorphisms in the regulation of IgE levels.

The human leucocyte antigen region

At chromosome 6p resides the human leucocyte antigen (HLA) region and the gene for tumor necrosis factor-α (TNF-α) as well. The HLA molecules are membrane-bound glycoproteins which bind processed antigenic peptides and present them to T-cells. Two HLA classes can be distinguished; class I is expressed on virtually every somatic cell; class II is merely expressed on B-cells, activated T-cells and monocytes/macrophages.

Polymorphisms in genes encoding the HLA class II molecules are associated with the specific IgE responses to several small allergens, such as ragweed pollen.[99] Other studies have failed to extend this finding to common major allergens, such as house dust mite.[100,101] Certain HLA class II alleles may be

important in susceptibility to isocyanate-induced asthma, the most common cause of industrial asthma.[102] In a collaborative US study, in Caucasian pairs of siblings with asthma, an increased sharing of alleles was found at chromosome 6p.[62] However, it is questionable whether the HLA region is implicated in asthma, considering that several other studies could not identify significant associations between asthma and the HLA region.[103,104] Thus, as atopy was present in over 75% of the US sample, the finding of linkage of asthma on 6p may, in fact, reflect the known association of the specific IgE response and the HLA region on 6p.

Tumor necrosis factor-α

TNF-α is a potent modulator of the immune inflammatory response and elevated levels can be detected in sputum and bronchoalveolar lavage fluid of patients with asthma during asthmatic attacks. Therefore, polymorphisms in this gene that may upregulate TNF-α production have been studied by Albuquerque *et al*.[105] In the promoter region of TNF-α on chromosome 6p, a polymorphism was detected at position 308 (G to A substitution), called the TNF1 allele. This polymorphisms could be associated with a six- to sevenfold upregulation in transcription of TNF-α. In a sample of 124 Australian schoolchildren, aged 6 - 12 years, this polymorphism resulted in a fivefold increased risk to asthma, defined as physician diagnosed asthma requiring the use of prophylactic medication. Moreover, all patients had positive skin prick to one or more common aeroallergens and a family history of asthma and/or atopic disease in first degree relatives.[105]
Within 7 cM of the TNF-α gene, a polymorphism in the first intron of the lymphotoxin-α gene (LTα*2 allele) showed a similar association. Furthermore, at chromosome 6p, the HLA region may also be involved in these atopic individuals. On the contrary, Moffat and Cookson found positive associations between asthma (questionnaire defined) and TNF2 and LTα*1 alleles.[106] This illustrates, that a positive association needs to be supported by linkage studies and functional studies, before definitive conclusions can be drawn regarding the role of these polymorphisms in the pathogenesis of asthma.

The cytokine gene cluster and other candidate genes

The cytokine gene cluster at chromosome 5q31-33 contains several pro-inflammatory cytokines (IL-3, IL-4, IL-9, IL-13), the glucocorticoid receptor, leukotriene C4 synthase (LTC4 syntase), CD-14 and several other candidate genes. Much interest has been given to cytokines that upregulate Th2-like lymphocytes, and therefore promote IgE production and airway inflammation. In general, studies in patients with asthma showed elevated IL4, IL-5, IL-9 and IL-13 production, and reduced IFN-γ production. These elevations may be due to polymorphisms that upregulate regulation of cytokine production. It is also possible that changes in other cytokine-genes or transcription factors that regulate these cytokines are responsible for these elevations. To date, there is some evidence for a possible role of a change in the promoter region of the IL-4 gene[107,108], the IL-9 gene[61,109], and the IL-13 gene[110], but not the IL-5 gene.[111]

The CD14 gene resides in the vicinity of the cytokine gene cluster on chromosome 5q and encodes for a high-affinity lipopolysaccheride receptor. This receptor is present as membrane bound CD14 on monocytes, macrophages and neutrophils, and in a soluble form (sCD14) in serum. Baldini and coworkers studied this CD14 gene based on the hypothesis that bacterial antigens could influence the Th1-Th2 balance and thus the development of atopy, through a CD14 dependent pathway. In a population study of children in the USA, a promoter polymorphisms in the CD14 gene was associated with levels of sCD14 in serum, total IgE levels and number of positive skin tests.[112] This interesting finding merits further study.

Other candidate genes for asthma and atopy include the IFN-γ gene at chromosome 12, T-cell-receptor genes at chromosome 7 and 14. A screening of the IFN-γ gene on chromosome 12 revealed no sequence variants in patients with asthma and controls.[113] Other groups have studied the genetics of the T-cell receptor. In most individuals, this receptor is made up of α-chains (gene at chromosome 14) and ß-chains (gene at chromosome 7). Around the α-chain, increased sharing of allele was found for the specific immune response in a UK and Australian population.[114] Around the ß-chain gene, increased sharing of alleles was found for childhood asthma and serum total IgE in a Japanese study [115] This study could not confirm the linkage to the region of the α-chain of the T-cell receptor gene. Further studies are needed to confirm these findings.

In summary, association studies of candidate genes have lead to interesting new insights into the genetics of asthma. Two polymorphisms of the $ß_2$-adrenergic receptor most likely do not cause asthma, but modify the severity of asthma. An interesting feature is that these polymorphisms might be involved in the response to medication. The high affinity IgE receptor could play a role in atopy and asthma in some populations. The E237G polymorphism of the high affinity IgE receptor especially needs to be studied in other populations. The IL-4 receptor a chain is a strong candidate for atopy, based on different association and functional studies. Finally, numerous other candidate genes have been studied. None of these candidate genes meet all four criteria as stated in the first paragraph of this section on candidate genes. It is clear that in the near future more genetic and functional studies are needed to clarify the role of these candidate genes in asthma and atopy.

Summary and future developments

The genetics of asthma has become a promising new field of research. In the pathogenesis of asthma multiple genes interact with each other and the environment. In different populations, different genes may have a major effect in the clinical manifestation of asthma. To date, several candidate genes

have been identified. Polymorphisms in the ß$_2$-adrenergic receptor do not cause asthma, but modify asthma into a more severe phenotype. Furthermore, polymorphisms in the ß-chain of the high affinity IgE receptor could play a role in a subset of the patients with atopy. However, most of the susceptibility genes for asthma and atopy remain to be determined. The recent identification of multiple linkages between asthma and different chromosomal regions represents a first step towards the identification of these asthma genes. However, in recent years, the procession from linkage to the actual identification of the gene has proved to be difficult.

In the coming years, future developments in molecular biology and genetic epidemiology may accelerate the process of the identification of genes for asthma and atopy. In the field of molecular biology, the Human Genome Project has the ultimate goal to sequence the human genome by 2003, and to identify single nucleotide polymorphisms throughout the genome. This project will aid genetic studies to a great extent.[116] Furthermore, one may anticipate new observations from animal studies, leading to further understanding of the genetics of human asthma. A genome-wide search for AH in mice has been completed, and has resulted in two possible linked regions on the mouse genome.[117] This approach could identify genes important in the regulation of AH in mice, and possible in men by searching for their homologous genes in human DNA.

In the field of genetic epidemiology, an interesting new method is the mapping of genes through the systematic analysis of shared haplotypes of affected individuals in founder populations. This approach is based on the idea that current asthmatics have identical copies of parts of chromosomes from a common ancestor. If one is able to identify such a chromosomal region which is identical by descent, it is likely that this chromosomal region contains an asthma susceptibility gene.[118]

The potential benefits of the identification of susceptibility or modifier genes for asthma are numerous. First of all, identification of persons at risk for asthma gives opportunity to early prevention, such as allergen avoidance or early introduction of medication. Secondly, protein products of these genes are potential drug targets, opening the way to causative rather than symptomatic treatment. Another clinical application may be that certain polymorphisms could result in a more severe asthmatic phenotype or predict resistance to therapy (pharmacogenetics). The latter is illustrated by the recent finding in a clinical trial of an experimental 5-lipoxygenase inhibitor in 325 patients with asthma. In this study, variants in the promoter region of the 5-lipoxygenase gene were associated with response to this anti-asthma treatment.[119]

In conclusion, although considerable progress regarding the genetics of asthma has been made, important questions remain to be answered. Which genes are the susceptibility genes for asthma? Are the genes for airway hyperresponsiveness and atopy the same or different? What is the biological function of asthma susceptibility genes? How do these genes interact with each other and with the environment? It will be a major challenge to unravel this complex genetic disease in the coming years.

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Chapter 2 | Defining asthma in genetic studies

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Summary

Genetic studies have been hampered by the lack of a gold standard to diagnose asthma. The complex nature of asthma makes it more difficult to identify asthma genes. Therefore, approaches to define phenotypes, which have been successful in other genetically complex diseases, may be applied to define asthma in genetic studies. These approaches include narrowing of the disease definition and use of intermediate phenotypes of asthma. Future studies are required to apply these approaches in genetic studies of asthma and, most likely, this will facilitate the search for genes for asthma.

Introduction

The definition of asthma has been the subject of ongoing debate, both in clinical[1] and asthma research[2] fields. Genetic studies of asthma have rekindled this debate.[3-5] There are three reasons to begin discussing the "best" definition of asthma in genetic studies. First, genetic methods, such as linkage studies, are sensitive to the misclassification of individuals. Misclassifying non-affected individuals as individuals with asthma reduces the statistical power of these genetic methods.[6] Second, replication of linkage results has been proven difficult in genetic studies of asthma. This may be explained by the fact that the definition of asthma and atopy differs between the various studies. As an example, atopy has been defined as a self-report of having an atopic disease (asthma, hay fever and eczema) in genetic studies, or as having (combinations of) elevated serum total IgE levels, positive skin prick tests or elevated serum specific IgE levels. Third, genetic research is hampered by the lack of a gold standard to diagnose asthma.
The aim of this paper is to discuss definitions of asthma. Furthermore, we will discuss applications of different definitions of asthma in research on its genetic background.

The definition of asthma

In previous decades, individuals and expert panels, as reviewed by Wiesch *et al.*,[6] made different definitions. A recent definition was published by the National Heart, Lung and Blood Institute in 1995: "*asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular mast cells, eosinophils, T-lymphocytes, macrophages, neutrophils and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial responsiveness to a variety of stimuli.*" [7]
Application of this definition does not lead to operational criteria, by which asthma can be diagnosed with 100% specificity and 100% sensitivity. It is therefore not guaranteed that false-positive and false-negative classifications are excluded. This will be briefly illustrated for three elements of the definition: symptoms, variable airway obstruction and bronchial hyperresponsiveness (BHR).
Symptoms consistent with asthma are also reported by patients with other pulmonary diseases. Wheeze and cough is often reported by non-asthmatic children, who have a viral respiratory tract infection. Wheeze is also a major symptom of individuals with chronic obstructive pulmonary disease (COPD). Airway obstruction, which can be detected in the majority of patients with asthma, can be absent in patients with mild and intermittent asthma. Furthermore, the reversibility of airway obstruction is lost in some patients

with asthma in older age.[8] This irreversible airway obstruction may be the result of airway remodelling in ongoing asthma. This makes the differentiation between asthma and COPD in elderly people sometimes difficult.
BHR is present in the majority of patients with asthma. It can be detected in 6-35 % of the general population. Thus, with approximately 6 % of the population having asthma, BHR is also present in asymptomatic individuals. The population prevalence of asymptomatic BHR varies from 2.2 to 14.3 % in various studies.[9] In addition, BHR can be detected in approximately two-thirds of smokers with COPD.[10]
It is unknown if measures of airway inflammation will bring a solution. Airway inflammation is regarded as the key pathophysiological process in asthma and is partly expressed in BHR. The performance of bronchial biopsies, and/or broncho-alveolar lavage to study airway inflammation is not feasible in large populations that need to be studied in genetic research. Finally, measurements of nitric oxide in expired air, another method to study airway inflammation, are variable in asthma. Elevations in expired nitric oxide levels have been reported for other diseases as well. Thus, genetic studies lack feasible and valid methods to assess airway inflammation.[11]
A diagnosis of asthma is called an asthma phenotype in genetic studies. The asthma phenotype can be assessed by questionnaires or can be built up by the use of measurements of clinical characteristics associated with asthma, such as BHR or airway obstruction. These characteristics are called intermediate phenotypes of asthma (table 1).
The question, how to define the asthma phenotype, is not easily answered. The goal of current genetic studies is to find genes for asthma: variants in genes that lead to a higher risk of asthma. Thus, a good definition of the asthma phenotype would be the definition that reflects the function of these genes for asthma. However, these genes are not known and no prediction about their function can be made. To overcome this limitation, different approaches can be applied that have been successful in defining a disease phenotype in other genetically complex diseases.[12]

The heterogeneity of asthma

To apply different approaches to define the asthma phenotype, investigators may consider the heterogeneity of asthma and the statistical power of genetic studies.

Heterogeneity

Asthma is a phenotypical heterogeneous disease. This is illustrated by the broad variations in the age at onset; the severity of asthma, from mild asthma to death of asthma; the progression of asthma, from the outgrowing of asthma to persistent asthma; the interrelationship between asthma and atopy and the response to asthma medication.
Asthma is also a genetical heterogeneous disease. It is now well accepted that the development of asthma involves multiple genes that interact with each

other and with the environment.[13] This is evidenced by the results of family studies, in which the inheritance of asthma could not be explained by a single gene model but rather by oligogenic or polygenic models. In addition, the published data of linkage studies in asthma point to different regions in the human genome, including chromosomes 5, 6, 11, 12 and 14.[13]

Statistical power

Each of these genes for asthma separately may confer only a small increased risk to asthma i.e. it has a low genotype relative risk (GRR). A GRR is the increased chance that an individual with a particular genotype has the disease. A low GRR has consequences for the power of genetic studies. In linkage studies, the power is the probability of correctly identifying a true linkage. The power of the affected sib-pair design and the association design was analyzed in a paper by Risch and Merikangas.[14] Under the assumption of a GRR of 2 and an allele frequency of 0.50, 2498 families are needed to achieve a power of 80 % to detect linkage using the affected sib-pair design. If the GRR is 4, the number of families needed drops to 297.[14] Thus, to identify genes of GRRs of 2 or less, an extremely large number of families is required for the affected sib pair design. Genetic approaches become more feasible if higher GRRs are considered. Thus, approaches that result in a rise of this genotype relative risk are needed. These approaches include narrowing of the disease definition and use of intermediate phenotypes.

Approaches to define an asthma phenotype

Narrowing the disease definition

The first approach is to narrow the disease definition. Four approaches to narrow the disease definition of genetically complex diseases were reviewed by Lander and Schork.[12]

1. The use of age at onset. In genetic studies of breast and prostate cancer, the selective inclusion of families with early onset of disease identified a subset of the population and in this subset significant linkage results were found.[15,16] In asthma, there is some evidence that the heritability of asthma, i.e. the proportion of the phenotypical variance that is due to genetic factors, decreases with age.[17] Thus, in genetic studies, it may be rewarding to include patients with asthma with an early age at onset.
2. The use of differences in the clinical phenotype. This strategy was successful in the identification of a gene for colon cancer in patients with extreme polyposis.[12]
3. The selective inclusion of individuals with a family history of the disease.
4. The use of a "severe" phenotype. In the case of asthma, patients with ongoing, severe, asthma may be especially interesting for genetic studies. However, the evidence that the severity of asthma is due to genetic factors is scanty.[18]

These approaches have not been explored in full detail in genetic studies of asthma, yet they may improve the prospects.

Table 1 Possible definitions of asthma and atopy in genetic studies

Asthma
- questionnaire
  - *symptoms (wheeze, dyspnea, cough, nocturnal symptoms)*
  - *self-reported (doctor diagnosed) asthma*
  - *use of medication for asthma*
- video questionnaire
- doctor's diagnosis

Intermediate phenotypes of asthma
- bronchial hyperresponsiveness
  - *direct (methacholine, histamine)*
  - *indirect (AMP, cold air challenge)*
- reversibility on $ß_2$-agonist
- variability of peak expiratory flow measurements
- lung function measurements (e.g. FEV_1, VC)
- total number of eosinophils in peripheral blood

Combination of questionnaires and intermediate phenotypes
- asthma score
- asthma algorithm

Atopy
- questionnaire
  - *symptoms of atopic asthma, rhinitis and dermatitis*
  - *self reported (doctor's diagnosed) atopic asthma, rhinitis or dermatitis*
  - *use of medication for atopy*
- doctor's diagnosis

Intermediate phenotypes of atopy
- serum total IgE
- serum allergen specific IgE
- allergy skin tests

Intermediate phenotypes

A second approach is to study intermediate phenotypes of asthma and atopy. Examples of intermediate phenotypes of asthma are BHR, peak flow variability and reversibility to a $ß_2$-agonist (table 1). As asthma is likely to be the end-stage of the action of multiple genetic and environmental factors, intermediate phenotypes may be due to the action of a limited number of genes. As an example, atopy may be due to multiple genes, including genes for the regulation and production of levels of total IgE, genes for the specificity of IgE and tissue specific expressed genes for end-organ responsiveness. When total IgE is studied separately, it may be easier to identify genes important in total IgE regulation. This can be illustrated by a study of 92 Dutch families, ascertained through a proband with asthma. In this study, a two locus segregation analysis of serum total IgE was performed. The first locus alone explained 50.6 % of the variance in total IgE, the second 19.0 % Taken together, these two loci account for 78.0% of the variability in serum total IgE levels.[19] This result also confers with the insight that even more than two genes are relevant in the regulation of high or low serum IgE levels. A drawback of the separate study of intermediate phenotypes is that genes of pleiotropic effects could be missed. These are genes that have effects on multiple phenotypes.

From intermediate phenotypes of asthma to asthma

Two different approaches have been used to combine reported symptoms and intermediate phenotypes to assess the complex asthma diagnosis: an asthma score and an asthma algorithm.

An *asthma score* is a quantitative score, in which asthma symptoms and intermediate phenotypes (BHR, FEV_1) are combined.[20] The asthma score is calculated as the first principal component of the results from questionnaires, the bronchial provocation test and FEV_1. This quantitative score is then used as a phenotype in genetic studies. Wilkinson *et al.* reported linkage of this phenotype to chromosome 12q.[20]

An *asthma algorithm* is a flow chart, which combines results from questionnaires and intermediate phenotypes. An example is an algorithm that was developed to classify family members of probands with asthma. This algorithm included BHR, pulmonary symptoms, smoking history, and (reversibility of) airway obstruction. Individuals were classified as having asthma, probable asthma, unclassifiable airway disease, COPD or unaffected. 265 First degree offspring of 92 patients with asthma were classified. 49 Individuals (18%) had definite asthma and 22 individuals (8%) had probable asthma. This study illustrated the familial clustering of asthma.[21]

The use of an asthma score or algorithm provides a reproducible way to diagnose asthma in participants of genetic studies. It should facilitate comparison of studies and pooling of data. This could improve the understanding of the genetics of asthma.

Conclusion

Genetic studies of asthma have been hampered by the lack of a gold standard. Asthma is a heterogeneous disease with variable age at onset, variable clinical expression and variable progression during a lifetime. The development of asthma involves multiple genetic factors that interact with each other and with environmental factors. This complex nature of asthma makes it more difficult to identify genes for asthma. Therefore, it may be advantageous to apply different approaches that have been successful in other genetically complex diseases to the field of asthma. These approaches include narrowing of the disease definition and use of intermediate phenotypes of asthma. Furthermore, scores or algorithms to define asthma may be used to make comparison and pooling of data a possibility. Future studies are required to study these approaches and, most likely, this will facilitate the search for genes for asthma.

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Chapter 3 | Genetics and environment in asthma: the answer of twin studies

(Eur Respir J 1999;13:2-4)

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Table 1 Results of twin studies of asthma

Population [reference]	Number of twin-pairs	MZ correlation+	DZ correlation+	Probandwise concordance MZ/DZ	Heritability	Comments
Swedish [5]	6996	0.65	0.25*			Adult population based study. Correlations calculated by Duffy et al.[2]
Ausralian [2]	3808	0.65	0.24*		0.60-0.75	"Asthma or wheezing" by questionnaire. Adult population.
Finnish [3]	13888	0.43	0.25*	0.13/0.07		Hospitalization, medication or cause of death / adult population based study.
Swedish [6]	434 M 456 F			0.62/0.26* 0.41/0.18*	0.36	Questionnaire (ever wheezing with short-ness of breath, wheezing without a cold, or parental reported asthma) / twins aged 7-9 years.
Norwegian [4]	2570	0.75	0.21*	0.45/0.12*	0.75	Population based study of twins aged 18 - 25 years.
Finnish [7]	1713	0.76	0.45	0.42/0.19*		Population based study of twins aged 16 years.
Danish [1]	1929 M	0.76	0.36	0.48/0.19*		Population based study of twins aged 12 - 26 years.
	2131 F	0.71	0.47	0.42/0.26*		Age 12-26.
	1867 M	0.81	0.37	0.51/0.16*		Age 27-41.
	2110 F	0.65	0.15	0.38/0.09*		Age 27-41.

MZ monozygous; DZ dizygous; M male; F female; + correlation is tetrachoric correlation; * statistically significant differences between MZ and DZ twin pairs

Twin studies have been widely used to estimate the genetic contribution to diseases. In this issue of the *European Respiratory Journal*, Skadhauge *et al.*[1] present the results of a large Danish, population-based twin study on asthma. In this study, the heritability in liability to asthma, i.e. the proportion of variance due to genetic factors, is estimated to be 0.77 for males and 0.68 for females. In other studies, the heritability of asthma is estimated to be between 0.36 and 0.75[2-7] (table 1). Thus, the results of this Danish study are consistent with those of other twin studies and add to the body of evidence indicating that the genetic contribution to asthma is considerable. In addition, the results of this study suggest individual specific, unshared, environmental factors to be important as well. In this editorial, the assumptions and methods of twin studies will be assessed, and the role of genetic and environmental factors in asthma reviewed.

Genetic studies using the twin-design have four major assumptions:[8]

1. Monozygous (MZ) and dizygous(DZ) twins are samples of the same gene pool;
2. twins are representative of the general population;
3. self-reported zygosity is correct in questionnaire based studies;
4. the environment for both MZ and DZ twins is similar.

The first and second assumptions are valid, provided representative or complete samples are taken from the population. The second assumption, the representativity, may not be totally valid because MZ and DZ twins differ from each other and from singletons with respect to their intrauterine environment.[9] The shared intrauterine environment may have an adverse effect on the growth and organ maturation of the foetus. However, this most likely does not influence the development of asthma, since the prevalence of asthma is comparable in twins and singletons.[1,2,4] The third assumption has been tested.[7] In general, self reported zygosity questions are adequate in 95-98 % of cases. Finally, the fourth assumption of an equal environment may not be valid in the case of asthma. For instance, it has been shown that MZ twins have more similar smoking patterns than DZ twins.[12] It is unknown if this higher similarity in MZ twins is also the case for other environmental factors, such as exposure to indoor allergens and viruses. A higher similarity in environment for MZ twins compared to DZ twins may lead to an overestimation of the heritability of asthma.

The method for diagnosing asthma is a self-reported questionnaire in most large scale twin studies. Subjects in these studies are not tested clinically. This method may lead to an under- or overestimation of asthma prevalence.[13, 14] Overestimation could occur, for instance, if asthma is diagnosed by questions on wheeze. Small children in particular may wheeze during the course of a respiratory infection, but not have asthma.[12] Therefore, studies on the genetics of asthma are currently directed at measurable clinical components of asthma, e.g. airway hyperresponsiveness (AH), reversibility and variability of airway obstruction. If it were known which of these components of asthma have a high genetic contribution, these

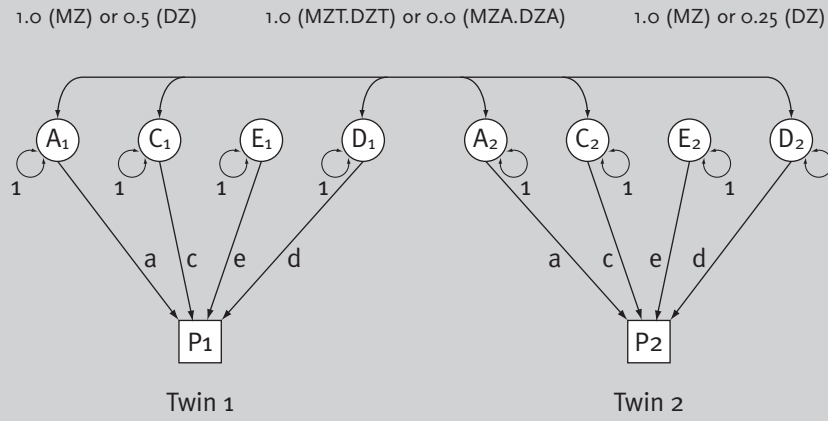
components can then be selected for genetic studies to find genes that regulate these components. As an example, one twin study of reasonable size on AH has been published, in which the heritability of AH to methacholine was 0.66.[16] Clearly, more studies are needed.

In general, statistical analyses of twin studies are complicated. In the study of twins, phenotypic similarities and differences are compared between MZ and DZ twins. MZ twins share 100% of their genetic information and DZ twins share on average 50%. If a trait is influenced by genetic factors, MZ twins should resemble each other to a greater extent than DZ twins, and the correlations between MZ and DZ twinpairs may be used to estimate the relative size of genetic and environmental influences.

In biometric modelling, one goes one step further. Since these biometric analyses are not frequently presented in pulmonary journals, we will first address some assumptions and methods before discussing the results of these analyses in the study of asthma. In biometric modelling, a quantitative genetic analysis is performed with dichotomous variables (e.g. asthma/not asthma). To permit these analyses, the first assumption is that disease status is determined by an unobserved continuous variable called the liability. If the liability falls above a threshold, individuals are classified as affected. The second assumption is that the distribution of the liability is normal.[17] The variance of the distribution of the liability is composed of multiple environmental and genetic influences. The environmental component can be dissected in influences shared by both twins and influences not shared. Furthermore, the genetic component consists of an effect of individual alleles on the trait (additive effect) or interaction between alleles at the same locus (dominance effect).[8] The last possible source of genetic effects, i.e. interaction of alleles at different loci (epistasis) can not be discriminated from dominant genetic effects in twin studies, which is a limitation of this design. Thus, the observed phenotypes P1 of twin 1 and P2 of twin 2 of a twin pair, will be linear functions of the underlying additive genetic influences (A-twin 1, A-twin 2), dominance genetic influences (D-twin 1, D-twin 2), shared environmental influences (C-twin 1, C-twin-2) and specific environmental influences (E-twin 1, E-twin 2). These functions are calculated in a path model by specialized computer programs (figure 1). The results of these calculations are then compared to known models, including a model in which the disease is caused by environmental factors alone, and a model in which the disease is caused by genetic factors, or combinations of these models. In this way, the best fitting model is calculated, i.e. the model that describes the data best. For more background information the reader is referred to the book by Neale and Cardon.[18]

By applying the above mentioned methods, Skudhauge *et al*.[1] found evidence for the liability for asthma in a model consisting of additive genetic factors and non-shared environmental influences, with modest evidence of effects of shared environment. Interestingly, other large-scale twin studies in different countries in the world came to the same conclusion.[2,4,7] The question that arises is: How do these findings relate to current evidence on the role of genetic and environmental factors in asthma?

Figure 1 Path diagram depicting genetic and environmental effects



Twin 1 and twin 2 are the first and second twin in a pair with phenotype P1 and P2, respectively. A (Additive genetic effects), C (shared environmental effects), E (non-shared environmental effects) and D (dominant genetic effects). a, c, d and e are path coefficients which are measures of variance of these genetic and environmental effects. MZ twins share 100% of their genes, therefore the correlation is 1 for additive and 1 for dominant effects. In DZ twins, these correlations are 0.5 for additive, and 0.25 for dominant effects.

Genetic factors

Major susceptibility genes for asthma and atopy have not been determined to date.[19] Several reports indicate a possible role for mutatations in a gene on chromosome 11 coding for the ß-chain of the high affinity IgE receptor in atopy or asthma, however, this picture is not clear as these mutations seem not to play in role in asthma nor atopy in other populations.[20] The gene coding for the $ß_2$-adrenergic receptor has been studied in more detail. Current data indicate that two common polymorphisms in this gene do not play a role in the causation of asthma. They may, however, modify asthma into a more severe phenotype expressed as more nocturnal complaints, higher use of inhaled corticosteroids in patients with these mutations and a reduced effect of ß-mimetics.[21]

Environmental factors

Environmental risk factors in asthma are active or passive smoking, exposure to allergens[22], viral respiratory infections, and possibly diet and air pollution.[23] Intuitively, most of these environmental factors appear to be largely shared. However, the twin studies suggest that not the shared, but the unshared individual-specific environment appears to be important. It is a challenge for researchers to assess which factors have these specific effects and to what extent the timing of exposure is relevant. It is of major interest to learn to understand how these factors interact with each other and with genetic factors.

In summary, what answers do twin studies give to the question which genetic and environmental factors cause asthma? First, twin studies have indicated the considerable genetic component of asthma. This component most likely consists of genes of additive effect. Second, twin studies have shown that individual specific environmental factors may be important as well. To further understand the genetics of asthma, we recommend to direct the twin approach at measurable components of asthma, such as airway hyperrresponsiveness, reversibility and variability in airway obstruction. Since we currently do not know which genes lead to susceptibility to asthma, the next challenge will be to study interaction of these genes and specific environmental factors in the development of asthma.

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Chapter 4 | Sibling effect on atopy in children of patients with asthma

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Abstract

Background. Multiple population studies have shown the presence of a sibling effect on atopic disease. However, it is unclear if the sibling effect is also of importance in subjects who are genetically at high risk for the development of atopy.
Objective. To study the presence of a sibling effect on markers of atopy (serum total IgE, specific IgE, skin tests) and asthma (bronchial hyperresponsiveness to histamine) in families ascertained through a parent with asthma.
Methods. First-degree offspring in 200 asthma families were studied (n=541). Mixed effects regression models were used to account for the dependence of the observations within a family, and to adjust for possible confounding variables.
Results. Multiple regression analysis showed that having older siblings was inversely related to atopy, defined as ≥2, ≥3, ≥4, or ≥5 skin tests (p=0.07–0.009). In addition, family size had a significant protective effect on the presence of specific IgE to common aeroallergens (p=0.03). Exposure to cigarette smoke in the first three years of life significantly increased the risk of having specific IgE to common aeroallergens (p=0.04). No sibling effect was detected for serum total IgE or bronchial hyperresponsiveness to histamine.
Conclusions. This study shows a protective sibling effect on the presence and severity of atopy but not on bronchial hyperresponsiveness in children who are genetically at risk. Exposure to cigarette smoke in the first three years of life is a risk factor for atopy irrespective of family size. The identification of the sibling effect in high-risk families stresses the need to understand the basis of this effect, in order to design future prevention programs.

Introduction

The increase in frequency of atopic diseases has urged the scientific community to identify factors that provoked this increase and to prevent further worldwide increases. Factors provoking this increase may be closely related to Western lifestyle, such as diet and the change in housing conditions leading to elevated levels of allergen exposure.[1] Identification of preventive factors of atopy development is of special interest, since this may lead to new strategies for disease prevention. Possible preventive factors include early childhood infections[2] or endotoxin exposure[3], early day care attendance[4,5], living on a farm[6,7], and a sibling effect.[8]

The sibling effect includes a protective effect of sibling order on atopic phenotypes, i.e. the number of older siblings, and/or an effect of family size, i.e. both older and younger siblings. Sibling effects on atopy have been identified in multiple population studies, in which atopy was defined by questionnaire[9-12], by allergy skin tests[13-16] and / or by measurements of specific IgE to aeroallergens.[17-19] Several studies have also identified a sibling effect on asthma and wheeze[5,20,21], but not on bronchial hyperresponsiveness.[13] Many previous studies have been performed in general populations, although the sibling effect has been included in two family studies. Consequently, published data on the interaction between atopic disease of various family members and the presence of the sibling effect in high risk families is limited. In 1997, Strachan predicted that families with a genetic predisposition '… may demonstrate steeper risk gradients with increasing number of siblings than surveys of the general population'.[22] This was partially confirmed by the study of Mattes et al., in which a sibling effect on atopy was seen in children of an atopic father, although not in children of an atopic mother. In contrast, results from the European Community Respiratory Health Survey showed no sibling effect in children from atopic parents, suggesting that environmental factors are less important in children with a strong genetic predisposition.[19] Since prevention programs are likely to be focused on children with a family history of atopy, it is important to know if environmental factors play a role in children with a strong genetic predisposition. Therefore, the aim of the present investigation is to identify a sibling effect on markers of atopy and asthma in offspring of parents with predominantly atopic asthma. In addition, we assessed whether parental smoking in the first three years of life of the child may explain the sibling effect.

Methods

Study population

Between 1962 and 1975, patients with asthma from the northern part of the Netherlands were referred to Beatrixoord, a regional asthma center in Haren, the Netherlands. These newly diagnosed patients with symptomatic

asthma who did not have an asthma exacerbation underwent a standardized, complete evaluation. For inclusion in the current study, at the time of initial testing all probands were younger than 45 years of age, had a doctor's diagnosis of asthma, and showed bronchial hyperresponsiveness (BHR) to histamine ($PC_{20} \leq 32$ mg/ml, 30 seconds inhalation protocol[23]. Between 1990 and 1999, 200 probands with asthma were restudied, together with their spouses, at least two children and available grandchildren aged 6 years or older. For the analysis presented in this paper, the first generation offspring of these families was selected. In this generation, every child had at least one parent with (mostly atopic) asthma, and data on family structure were available. Every child was assigned a sibling order according to the date of birth. Twins were assigned the same number equal to the number of the second twin. In addition, sibling size was recorded. Paternity status was verified by genotyping over 300 DNA markers for the genetic studies.[24]

Clinical and laboratory evaluation

All individuals answered a modified version of the British Medical Council questionnaire on respiratory symptoms, housing conditions and smoking. Questions on medication use were added. Smoking history was recorded by asking each subject the number of cigarettes smoked per day for every year of their life. From this, smoking during pregnancy was determined as well as exposure to cigarette smoke of their children during the first three year of life.

All individuals underwent spirometry, bronchodilator reversibility to 800 mg of inhaled albuterol (salbutamol), and bronchial responsiveness testing to histamine using a 30 second inhalation protocol previously described[23] Bronchial hyperresponsiveness was defined as a provocative concentration (PC_{20}) ≤ 32 mg/ml (30 seconds method).

Skin tests were performed in adults by intracutaneous tests and in children with skin prick tests, with a positive and negative control. For the current analysis, ten allergens used both in adults and children were selected: mixed grasses, tree pollens, and weeds, house dust mite, animal dander of dog, cat, horse, a mixture of guinea pig and rabbit, and the moulds Aspergillus Fumigatus and Alternaria Alternata (ALK-Abelló, Nieuwegein, the Netherlands). The maximum diameter and the perpendicular diameter of the wheal size were recorded after 15 minutes. An intracutaneous skin test was considered to be positive if the mean wheal diameter was ≥ 5 mm; a skin prick test was considered positive if the mean wheal diameter was ≥ 3 mm. The skin tests were not used for further analysis if the negative control gave a positive reaction.

Total IgE was measured by solid phase immunoassay in the first 92 families. The mean of two duplicate tests of IgE was used, and measurements were repeated if the difference between duplicates was > 5%. (Pharmacia, Uppsala, Sweden). In the second set of 108 families, serum IgE levels were measured by an enzyme linked fluorescent assay (Mini Vidas, Biomerieux Inc., Marcy, France). Specific IgE was measured by an in vitro test system

(Pharmacia CAP system, Phadiatop FEIA) according to the instructions of the manufacturer (Pharmacia Diagnostics AB, Uppsala, Sweden). In this assay, IgE is measured against a mixture of inhalant-allergens and is used as a general assessment of allergic responsiveness. It determinates the presence of specific IgE to the following antigens: house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), timothy grass (*Phleum pratense*), birch (*Betula verrucosa*) and olive pollen (*Olea europea*), two different weeds (*Artemisia vulgaris* and *Parietaria officinalis*), a mould (*Cladosporium herbarum*), and finally cat, dog and horse dander. The test was regarded positive if the fluorescence score of the subject's serum was higher than that of the reference as provided by the manufacturer. Finally, eosinophils were counted in a counting chamber. All participants were asked to stop asthma and allergy medication before the clinical testing if possible: specifically inhaled corticosteroids were stopped for 14 days, inhaled long acting beta-mimetics and oral antihistamines for 48 hours, inhaled short acting beta-mimetics and anticholinergics for 8 hours. Asthma patients did not have an asthma exacerbation or a course of oral prednisone in the 6 weeks prior to the study.

Informed consent

The study was approved by the Medical Ethics Committee of the University Hospital Groningen. In addition, the study was approved by the Institutional Review Board of the University of Maryland and Wake Forest University. Written informed consent and written parental consent were obtained from all participating adults and children, respectively.

Statistical methods

Serum total IgE was log transformed. Both parametric (T-test, ANOVA) and non-parametric analyses (Mann-Whitney, Kruskal Wallis test) were used to study differences in sibling order, depending on the normality of the distribution of the variables using SPSS 10.0. Sibling effects on atopy and BHR were assessed using mixed-effects regression models containing fixed effects in addition to random effects. These models adjust for the dependence of the observation within one family.[25] The sibling effect was adjusted for age, sex, and smoking habits. In addition, parental atopy (the presence and the severity of atopy), passive smoking in the first three years of life (measured by smoking of the parents during that period), maternal age at birth, and having pets or outdoor animals, were included in the model one by one. The latter variables only stayed in the model when they were significantly related to one of the markers of atopy or bronchial hyperresponsiveness in the child or when they influenced the effect of sibling order or family size. Models with sibling order and family size were run, and the best fitting models were used for further analysis. In addition to assessment of the sibling effect on the presence of positive skin tests and BHR, the sibling effect on severity of skin test positivity and bronchial responsiveness was assessed. This was done by using different cut-off values: ≥2, ≥3, ≥4, and ≥5 positive skin tests and $PC_{20} \leq 16$, 8 and 4 mg/ml histamine.

Table 1. Clinical characteristics of probands, spouses and their first degree offspring in 200 Dutch families

	First degree offspring	Probands	Spouses
Number	541	200	201*
Age, mean (SD), years	24.2 (9.2)	52.1 (8.4)	51.0 (9.2)
Male, %	45.2	62.0	37.8
Total IgE, geometric mean, kU/l (range)	62.7 (0 – 3360)	93.0 (1.0 - 2880)	26.2 (0.5 - 1940)
≥ 1 positive skin test, %	50.1	81.9	31.0
Specific IgE, % positive (Phadiatop)	43.9	72.4	15.1
FEV_1 % predicted pre medication (mean, SD)	93.8 (12.3)	69.6 (24.5)	98.4 (14.1)
FEV_1 % predicted post medication (mean, SD)	100.3 (11.9)	82.4 (23.5)	103.9 (13.7)
BHR to histamine, % $PC_{20} \leq 32$ mg/ml	46.3 #	88.2 #	25.6

* One proband married twice. Missing data due to following reasons: Not enough blood for serum total IgE measurement in 2 children, and for specific IgE measurements in 13 children; skin tests refused by 4 children. Spirometry before and after medication could not be performed reproducible in two children.
# BHR Bronchial hyperresponsiveness to histamine. Thirty probands and eight children were not tested due to an FEV_1 that was too low to be tested safely; or FEV_1 measurements that were not reproducible

Results

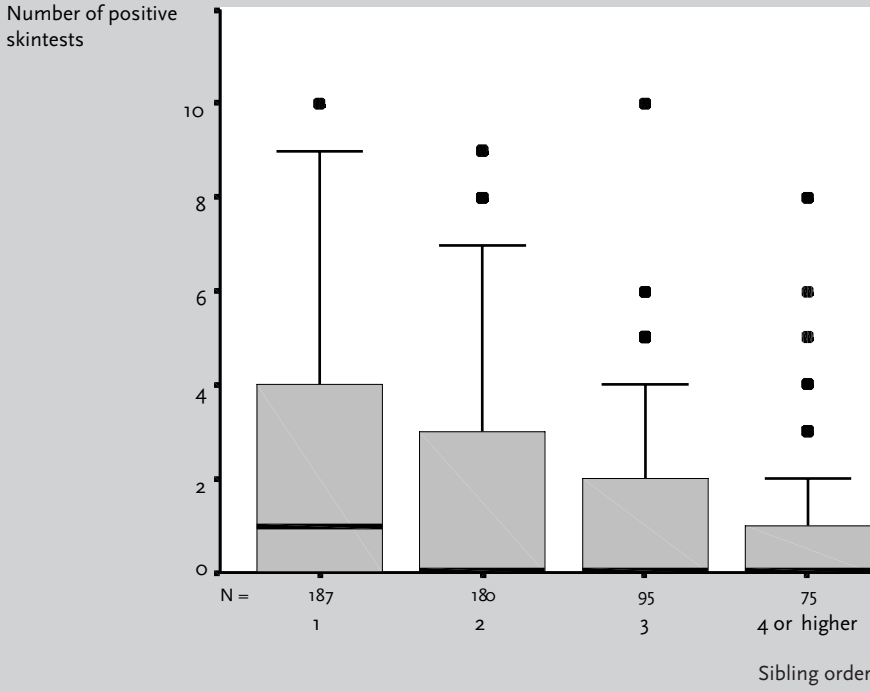
Study population

In the 200 families, 586 children were 6 years or older. Of these 586 children, 541 (92.3 %) were investigated. Reasons for non-participation were living abroad (n=5), deceased/disappeared (n=2), and refusal (n=38). In the first-degree offspring, one case of non-paternity was identified and paternal status was set to unknown. Baseline characteristics of the first-degree offspring, the probands and the spouses are shown in table 1. The majority of the first-degree offspring was adult, the mean age was 24 years. Half of the children were atopic, as detected by one or more positive skin tests (50 %) and 44% had specific IgE to common aeroallergens. In addition, 46 % of the children had bronchial hyperresponsiveness to histamine.

Sibling effect – descriptive analysis

Mean ages were significantly different based on sibling order 1, 2, 3, and ≥4 (table 2). Specifically, first-born children were older than second and third born children ($p<0.05$). First-born children had the highest prevalence of one or more positive skin tests (55%), specific IgE to common aeroallergens (46%) and bronchial hyperresponsiveness (50%). We observed lower prevalence rates for the consecutive siblings; the lowest rate was seen in the group of children born fourth or later: 43% had one or more positive skin tests, 34% had specific IgE to common aero-allergens and 38% had bronchial hyperresponsiveness. A similar trend was observed for geometric mean serum total IgE levels ranging from 67.3 kU/l in first-born children to 54.6 in children born 4th or later. Finally, the mean number of positive skin tests decreased significantly, with sibling order being 2.2 in the first-born to 1.2 in the last (≥ 4) born children (figure 1).

Figure 1. Number of positive skin tests for every birth order group.



Boxplot shows medians (bars) and interquartile ranges. Dots are outliers.

Skin tests

In the multivariate models, sibling order fit the data better than did family size. Different cut-off levels for skin test positivity were assessed: ≥1, ≥2, ≥ 3, ≥4, and ≥ 5 skin tests. When atopy was defined as at least one positive skin test, having one or more older sibling was not significantly related to atopy. However, when more stringent definitions of atopy (≥ 2, ≥3, ≥4, and ≥5 positive skin tests) were used in the same model, sibling order was significantly and inversely associated with severe atopy. Table 3a shows the results for skin test positivity defined as two or more positive skin tests. The sibling effect on severe atopy was confirmed by analysis of higher cut-off values of skin test positivity, i.e. siblings with higher birth order are less likely to have ≥ 2, ≥3, ≥4, and ≥5 positive skin tests than first-born siblings (table 3b). Possible confounding variables, such as the presence of indoor pets, the presence of outdoor cattle, smoking during pregnancy, current (passive) smoking, passive smoking during the first three years of life of the child, and maternal age at birth of the child were not associated with skin test positivity, nor influenced the relationship of sibling order with skin test positivity.

Table 2. Markers of atopy and asthma in children of patients with asthma stratified on birth order

	Sibling order			
	1	2	3	≥ 4
Number	188	182	96	75
Age (mean, SD) *	25.8 (8.9)*	23.2 (9.3)*	23.0 (9.1)*	25.5 (9.7)
Male, %	39.3	47.3	51.0	48.0
Serum total IgE, kU/l geometric mean (range)	67.3 (1.0-3360)	61.1 (0-3040)	60.3 (1.0-1490)	54.6 (1.0-2000)
Skintests				
≥ 1positive, %	54.5	48.9	49.5	42.7
≥ 4 positive, %	29.7	22.2	18.9	12.0
Number of skin tests positive (median, IQR~)	1 (0-4) #	0 (0-3) #	0 (0-2) #	0(0-1) #
Specific IgE, % positive	46.2	46.0	43.0	34.2
Bronchial hyperresponsiveness: $PC_{20} \leq 32$ mg/ml, %	50.2	46.9	44.2	37.8

* P<0.05 sibling order 1 versus 2 and 3

~ IQR interquartile range

Significant differences between four sibling order groups, Kruskal Wallis test (p< 0.05)

Table 3a Sibling order effect on skin test positivity (≥ 2 positive skin test)

Variable	Estimate	Standard error	P
Intercept	-4.27	0.71	0.000
Male sex	0.32	0.32	0.311
Smoking habits			
- ex-smoker	0.01	0.48	0.98
- smoker	0.20	0.34	0.55
Age (quartiles) *			
- >17 – 24 years	**1.23**	**0.43**	**0.004**
- > 24 – 30 years	**1.87**	**0.52**	**0.000**
- > 30 years	**0.42**	**0.50**	**0.392**
Skin tests parent			
- Number of positive skin tests father	0.08	0.07	0.24
- Number of positive skin tests mother	-0.04	0.08	0.60
Siborder	**-0.33**	**0.14**	**0.018**

Skin test positivity corrected for bronchial hyperresponsiveness, serum total IgE levels and eosinophil count.
* reference category: ≤ 17 years.

Table 3b Sibling order effect on skin test positivity using different cut-off values for the number of positive skin tests

Number of positive skin tests	Estimate of siborder	Standard error	P value
≥ 1	-0.08	0.14	0.532
≥ 3	**-0.35**	**0.34**	**0.009**
≥ 4	**-0.28**	**0.16**	**0.070**
≥ 5	**-0.36**	**0.17**	**0.034**

Reference categories are <2, <3, <4 and < 5 positive skin tests, respectively. The estimates are adjusted for age, sex, smoking, number of popsitive skin tests of father and mother, bronchial hyperresponsiveness, serum total IgE, and eosinophil count.

Specific IgE to common aeroallergens

In the multivariate models, using family size described the data better than did sibling order. Family size was significantly and negatively related to the presence of specific IgE to common aeroallergens (table 4). Thus, for larger families, the prevalence of specific IgE to common aeroallergens in the children is lower. In addition, exposure to cigarette smoke during the first three years of life was associated with having positive specific IgE to common aeroallergens.

Table 4 Family size effect on specific IgE to common aeroallergens (Phadiatop)

Variable	Estimate	Standard error	P
Family size	**-0.31**	**0.14**	**0.03**
Male sex	**1.12**	**0.41**	**0.007**
Age (tertiles) *			
- **19-28 year**	1.03	**0.53**	**0.05**
- > 28 year	0.37	0.51	0.47
Smoking habits			
- ex-smoker	0.25	0.48	0.63
- smoker	-0.25	0.40	0.54
Passive smoking in first three years of life	**1.04**	**0.52**	**0.04**

Corrected for bronchial hyperresponsiveness, serum total IgE levels and eosinophil count.
* Reference category is ≤ 19 years

Bronchial hyperresponsiveness to histamine and serum total IgE levels

Sibling order and family size were not associated with the presence of bronchial hyperresponsiveness in a multivariate model after adjustment for age, sex, smoking, and eosinophil counts (sibling order: estimate 0.09, standard error (s.e) 0.13, p=0.48). Similar results were observed when more severe cut-off levels for BHR were taken (PC20 ≤ 16, 8 or 4 mg/ml). In addition, sibling order and family size were not associated with serum total IgE levels (reference category first-born: second born: estimate -0.03, s.e. 0.05, p=0.62; third-born estimate 0.0, s.e. 0.07, p=0.96; ≥ fourth born estimate 0.0; s.e. 0.08, p=0.97).

Discussion

This study shows that in families with a parent with asthma, children with older siblings are less likely to have positive skin tests (defined as ≥ 2 skin tests), whereas children with more siblings (both older and younger) are less likely to have positive specific IgE to common aeroallergens. Furthermore, children who were exposed to passive smoking during the first three years of life were more likely to have elevated specific IgE levels. However, we found no sibling effect on serum total IgE and bronchial hyperresponsiveness in children from patients with asthma. Environmental factors as detected by the sibling effect may modify the outcome of atopy but not of bronchial hyperresponsiveness in children who are at high risk due to genetic predisposition.

We observed a significant protective effect of a larger family size on the presence of specific IgE to common aeroallergens. This confirms several reports showing a similar effect in population studies and in one study investigating 1440 families.[9,17-19] In the latter study, a sibling effect was present in children of atopic fathers, but not of atopic mothers. Families were investigated with questionnaires, and paternal atopic status was assessed by asking one parent, generally the mother. Thus, bias could have been introduced in that study if only more severe atopic fathers were reported. In addition, recall bias may influence the results of questionnaire based family studies. This has been recently shown in the MAS study, in which self reported atopy by the parents was influenced by current atopy of the child.[26] We studied objective markers of atopy, such as skin tests and specific IgE to common aeroallergens and found effects in families with atopic asthmatic fathers and / or mothers. Differences in results may also be explained by methods of family ascertainment. In the present study, ascertainment was based on asthmatic status of one of the parents, and a high percentage (> 92 %) of all children were studied. Therefore, it seems unlikely that selection bias may have confounded our results.

Our findings appear to be in contrast with the results from the European Community Respiratory Health Survey (ECHRS). In this survey, a sibling effect was present in children of non-atopic parents, but not in children of atopic parents. However, the severity of atopy was not taken into account, as atopy was defined as the presence of one or more positive skin tests.[19] In contrast to the ECRHS, we investigated offspring of predominantly atopic asthmatic probands and found a protective effect of sibling order on skin test positivity, yet only when we defined a more severe atopic phenotype. We speculate that although genetic predisposition in these children may lead to skin test positivity, the sibling effect appears to modify the severity of atopy (i.e. smaller number of positive skin tests). The sibling effect on severe atopy has also been indicated by an Italian study of recruits, which defined severe atopy by a higher cut-off point for specific IgE to common aeroallergens.[18] In addition, in a German population of children, the sibling effect on atopy was studied by assessment of skin test positivity. In this study, the sibling effect appeared to be present with higher cut-off values for the mean diameter of the skin test (i.e. ≥4 or ≥5 mm), but not with lower values (≥ 2 mm).[14] For skin test positivity and specific IgE to common aeroallergens, different models fit the data best for sibling order and family size. The trends in the data were similar (data not shown) and we have chosen the best fitting models in our multivariate analyses. The sample size of our study did not allow us to further disentangle effects of size and order, or effects of the gender of the older siblings.[8] Moreover, since we only ascertained families with at least two siblings, the children at highest risk for atopy (one child per family) were not included in our study. Thus, we conclude that given the relatively small sample size the sibling effect is an important environmental factor acting in these high-risk families.

Our study did not find a sibling effect on serum total IgE levels and bronchial hyperresponsiveness. Similar results have been observed in other studies.[13,17,19] It appears that environmental factors are less able to modify serum total IgE levels than specific IgE levels or skin test positivity. This is consistent with genetic studies in our families, showing high heritability estimates of serum total IgE levels of 55 %, compared to 41% for specific IgE to common aeroallergens and 25% to skin tests.[24] No sibling effect was observed on bronchial hyperresponsiveness. Other studies have identified a sibling effect on doctor diagnosed asthma[5] or self-reported asthma symptoms in children.[20,21,27], although the number of studies supporting a role on hayfever and atopic sensitization appear to be larger.[10,11,14-16,18,19,28,29] We cannot exclude that this study was not powerful enough to show a significant sibling effect on bronchial hyperresponsiveness. However, other explanations, such as differential environmental effects on asthma and atopy need to be considered.

Strachan hypothesized that the sibling effect could be explained as a protective effect of childhood infections on the development of atopy.[12] Children who grow up in larger families report more childhood infections, such as prolonged colds.[30] This is supported by immunological data, indicating that infections may have a skewing effect on the Th1–Th2 balance towards the Th1 pathway.[31] Alternative explanations for the sibling effect, such as changing smoking patterns in the parents have also been assessed. Parents could respond to the presence of allergy in the first child and change their smoking behaviour for the consecutive children leading to lower prevalences of atopy in these children. Although passive smoking in the first three years of life was associated with the presence of specific IgE to common aeroallergens, it did not appear to be associated with sibling order.

It has been suggested that the sibling effect is an interaction of genes with environment (infections).[32] Further studies of genes that respond to these infections are needed. Interesting candidate genes are genes that encode for receptors of lipopolysacherides or other bacterial wall components, such as CD14[33,34]and the Toll like receptor 4.[35] A precise understanding of protective effects of infections may lead to new preventative measures, that may possibly be applied in high-risk families.
In summary, this study shows a sibling effect on the presence and severity of atopy, defined by specific IgE to common aeroallergens and skin test positivity in families at high risk for atopy and asthma. Furthermore, an independent negative effect of smoking during the first three years of life on atopy was observed. The identification of the sib effect in high-risk families stresses the need to understand the sibling effect, in order to design better preventive programs.

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Chapter 5 | Major genes regulating total serum IgE levels in families with asthma

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ABSTRACT

Immunoglobulin E (IgE) has a major role in the pathogenesis of allergic disorders and asthma. Previous data from 92 families, each identified through a proband with asthma, showed evidence for two major genes re gulating total serum IgE levels. One of these genes mapped to 5q31-33. In the current study, the segregation analysis was extended by the addition of 108 probands and their families, ascertained in the same manner. A mixed recessive model (i.e. major recessive gene and residual genetic effect) was the best fitting and the most parsimonious one locus model from the se gregation analysis. A mixed two-major gene model (i.e. two major genes and residual genetic effect) fit the data significantly better than did the mixed recessive one-major gene model. The second gene modified the effect of the first recessive gene. Individuals with the genotype aaBB (homozygous high-risk allele at the first gene and homozygous low-risk allele at the second locus) had normal IgE levels (mean 23 IU/ml) and only individuals with genotypes aaBb and aabb had high IgE levels (mean 282 IU/ml). A genome-wide screen was performed using variance component analysis. Significant evidence for linkage was found for a novel locus at 7q with a multipoint lod score of 3.36 ($p = 0.00004$). A lod score of 3.65 ($p = 0.00002$) was obtained after genotyping additional markers in this region. Evidence for linkage was also found for two previously reported regions, 5q and 12q, with lod scores of 2.73 ($p = 0.0002$) and 2.46 ($p = 0.0004$), respectively. These results suggest that several major genes, plus residual genetic effects, regulate total serum IgE levels.

INTRODUCTION

Immunoglobulin E (IgE) has important functions in the development of allergic disorders and asthma. High total serum IgE levels have been reported to be correlated with the clinical expression of allergy and asthma.[1-4] Epidemiologic studies have shown that higher IgE levels are associated with bronchial hyperresponsiveness (BHR), a major component of the asthma phenotype.[2, 5, 6] In fact, high total serum levels IgE predicts the development of asthma independent of other allergic factors. Therefore, understanding the genetic mechanisms regulating total serum IgE levels will be important in efforts to dissect the hereditary components of asthma and allergy, complex genetic disorders influenced by the interactions between multiple genes and environmental exposures.[7]

In a study reported elsewhere, our use of two-locus segregation analysis revealed evidence for two major genes and a residual genetic effect regulating total serum IgE levels in the first set of 92 Dutch families ascertained through a parent with asthma.[8] For the first of these loci, evidence of linkage to chromosome 5q was obtained from both one-locus and two-locus analyses based on a candidate gene approach.[8, 9] Now that data are available on the total sample of 200 families ascertained through a parent with asthma (all of which originally had been characterized approximately 25-35 years previously), we have performed a genomewide search for genes regulating total serum IgE levels. The purposes of the current study are: 1) to determine the relationship between total serum IgE levels and other measures of asthma and allergy, 2) to examine the familial aggregation of the total IgE levels by estimating the degree of correlation between relative pairs and to test the fit of various models by performing one- and two-locus segregation analysis, 3) to systematically search for the locations of the major genes across the genome with evenly spaced autosomal markers (~10 cM apart) and variance component analyses. High total serum IgE levels, an important phenotype closely associated with asthma and allergic disorders, are an ideal quantitative trait for use with this analytic approach.

FAMILIES AND METHODS

Family ascertainment

A total of 200 families (1,171 family members) were ascertained through probands who were initially studied during 1962-75 at Beatrixoord Hospital, Haren, the Netherlands, a regional referral center for patients with asthma and other airway obstruction diseases. Patients who had symptomatic asthma without a current asthma exacerbation were referred to this hospital and were admitted for a standardized, complete evaluation. At the time of initial testing, all probands had asthma symptoms, were hyperresponsive to histamine (PC_{20} forced expiratory volume in 1s [FEV_1] $\leq$ 32 mg of histamine/ml, 30-s method), and were < 45 years old. The first 92 probands, together with their spouses, children, children's spouses, and

grandchildren > 6 years old, were recruited and evaluated in the early 1990's.[10] To enlarge the sample, 108 probands and their families were collected during 1994-1999 by use of the same ascertainment scheme that was used for the first 92 families. This study was approved by the Medical Ethics Committee of the University Hospital Groningen, and all participants signed an informed-consent docuement. Among the 200 families, 166 families consisted of two generations, 33 consisted of three generations; 1 family consisted of four generations.

Clinical evaluation

All participants answered a modified British Medical Society Respiratory Questionnaire, as well as additional questions pertinent to the diagnosis and assessment of asthma and obstructive pulmonary disease. Pulmonary function was tested using standard methods that included spirometry before and after the administration of inhaled salbutamol (800 mg). Testing of bronchial responsiveness to histamine was performed using the method of De Vries et al.[11], which had been used to assess the initial participants during the period 1962-75. The reactivity-testing protocol consists of ha ving the subject inhale increasing concentrations of histamine, for 30 se conds of tidal breathing up to a maximum dose of 32 mg of histamine/ml. The test was stopped if FEV_1 decreased ≥ 20%. Other evaluations included skin tests for responsiveness to 16 common allergens (intracutaneous tes ting in adults and prick testing in children), a differential blood count (including total eosinophil count), and measures of total serum IgE levels as well as IgE specific to house dust and mixed pollens. A positive skin test was defined as the presence of ≥1 reaction with a wheal diameter ≥ 5mm. Total serum IgE was measured by solid phase immunoassay (Pharmacia IgE EIA: Pharmacia Diagnostics).

Molecular methods

Blood samples were shipped from the Netherlands to the molecular genetics laboratory at the University of Maryland at intervals of ~ 2 wk. DNA was isolated by standard protocols using a Puregene kit (Gentra). For the genome wide screen, we used the Weber (version 8) set of markers, which spans the human genome at an average of 10 cM and consists of 366 autosomal markers, 86% of which are tri- and tetra-nucleotide repeats with an average marker heterozygosity of 76%. We performed multiplex PCR using fluorescently labeled primers, separated the resulting amplified fragments on denaturing polyacrylamide gels, detected the fragments with the use of ABI 377 sequencing machines, and scanned and scored the genotypes, using ABI software. A modified version of the program Linkage Designer[12] was used to bin the alleles and check inheritance. The output from Linkage Designer was then analyzed further, for any inconsistencies by operating the LINKAGE software without disease information. As a final check of the data, we used CRIMAP[13] to determine the order and length of the chromosomal map and to detect double recombinants.

Statistical methods

Total serum IgE levels were logarithm transferred ($\log_{10}$) in order to appro ximate a normal distribution (all analyses were performed using $\log_{10}$ (IgE) levels). Because log levels were higher in males and in younger individuals, the effects of sex and age was included in the genetic analyses. In the variance component analysis, adjustment of the fixed effects of sex and age was performed simultaneously whit fitting of the various models. To estimate the correlation coefficients of IgE for pairs of relatives and for complex segregation analysis, the fixed effects of age and sex were removed by taking the residuals after linear-regression analysis had been performed.

Correlation coefficients of total serum IgE levels among various pairs of re latives were estimated from the sums of squares and cross-products from the pairs, using the computer program FCOR of S.A.G.E. (Statistical Analysis for Genetic Epidemiology). Three distinct weighting methods were used. In the method of equal weight to pairs, every possible pair has equal weight. The pedigrees with large number of pairs will contribute more information than will pedigrees that contain a relatively small number of pairs. In the method of equal weight to pedigrees, each pedigree, regardless of its size contributes equal weight. The data are averaged within pedigrees before they are averaged across pedigrees. In the method of equal weight to nuclear families, only the nuclear families (parents and children) are included.

Complex segregation analyses assuming one- and two-locus models were used to evaluate the transmission of total IgE levels within the 200 families. An ascertainment correction was not used, for the following reasons: 1) the families were ascertained through a parent (not a child) with astma, not through probands with specific IgE levels; 2) the probands tend to have high IgE levels but with a large range of values; 3) we are interested in major genes in the specific population of families with asthma. In the one-locus segregation analysis, various models were evaluated, including a ge neral model and Mendelian major-gene models, an environmental model, a polygenic model, and mixtures of various polygenic models with either a major-gene model or an environmental model. The likelihoods that each of these models fit the observed family data were computed using the computer software package Pedigree Analysis Package (PAP), revision 4.0. In the cases of mixed models, the likelihoods were approximated by allowing information from previously analyzed family members to represent information about the entire family.[14] The parameters in the general models include (1) one allele frequency q_a (corresponding to a high value) and three genotypic frequencies (F_{AA}, F_{Aa}, and F_{aa}) that were assumed to occur in Hardy-Weinberg equilibrium; (2) three arbitrary transmission probabilities τ_{AA}, τ_{Aa}, and τ_{aa}, representing the probability that an individual of a given genotype transmitted allele A to the offspring and (3) three arbitrary genotypic means μ_{AA}, μ_{Aa}, and μ_{aa}, a common variance for all the genotypes, and a residual genetic heritability h^2, which is partitioned from the variance and represents the additive effects of polygenic loci. In the Mendelian models, the three transmission probabilities were fixed to the Mendelian

inheritance, ie. $\tau_{AA} = 1.0$, $\tau_{Aa} = 0.5$ and $\tau_{aa} = 0$. In the environmental models, the three transmission probabilities were set to be the same $\tau_{AA} = \tau_{Aa} = \tau_{aa}$, reflecting the independence between parental genotypes and the transmission probabilities. For the Mendelian-only models and environmental only models, h^2 is set to 0. For the mixed models, h^2 is estimated. Within the Mendelian models, a dominant model is derived by setting $\mu_{Aa} = \mu_{aa}$ and a recessive model is inferred by fixing $\mu_{AA} = \mu_{Aa}$.

Two-locus segregation analysis was performed using PAP. Analysis was performed to determine whether two major genes, compared to two environmental factors or one major gene, better modeled the segregation of IgE levels in the families. The parameters in the two-locus segregation analysis included: allele frequencies at each locus (q_a and q_b), the recombination fraction between the two loci (θ), the means (μ's) for the nine possible distributions representing the nine types of individuals (AABB, AABb, Aabb, AaBB, AaBb, Aabb, aaBB, aaBb, and aabb) with a common standard deviation (s), and a heritability (h^2), which is a measure of residual variance within each type. Mendelian models were inferred by setting the nine transmission probabilities (probability that an individual of a given genotype transmits allele A and B to his or her offspring) to Mendelian expectations. The environmental model was inferred by setting all nine transmission probabilities to equal values, reflecting the independence between parental genotypes and the transmission probabilities.

Two criteria were used to compare these models. For hierarchical models, the likelihood-ratio test was used. Twice the difference in likelihoods (-2lnL) between a restricted and an unrestricted model approximately follows a chi-square statistic, with degrees of freedom equal to the difference in the number of parameters used in the two models. The best fitting model is the one requiring the fewest estimated parameters, while giving a ln-likelihood not significantly smaller than the unrestricted model. When comparing non-hierarchical models, the Akaike's[15] Information Criterion (AIC) was used, $-2\ln L + 2k$, where k is the number of parameters estimated in the models. By this criterion, the most parsimonious model is the one with the smallest AIC score.

To estimate the effect of a major gene, we used genotypic probability estimators (GPEs)[16,17], as implemented in PAP. The genotypic probability (p_{ij}, the probability that individual i carries genotype j), equals the likelihood conditioned on individual i carrying genotype j, divided by the unconditional likelihood and computed with the parameters set at the maximum-likelihood estimates (MLEs). The mean of a trait Y for genotype j is calculated as $\mu_j = \Sigma P_{ij} / n_j$, where y_i is the IgE measured on person i and $n_j = \Sigma P_{ij}$. A t-test was used to determine statistical significance.

Variance-component linkage analysis was used to estimate the proportion of the variance attributable to residual genetic effects, random environmental effects, and quantitative trait loci (QTL). By fitting various models, it is possible to make inferences regarding the localization (the chromosomal regions mapped by the genetic markers, i.e. linkage) and the magni-

tude of effect sizes of major genes. Analyses were performed using the computer program package Sequential Oligogenic Linkage Analysis Routines (SOLAR),[18] which uses the computer program FISHER and SEARCH[19] for likelihood optimization in quantitative-trait analysis. For model fitting, the fixed effect of the covariates sex and age were removed by simultaneously including them in the models. In the most basic model, the expected covariance matrix for a pedigree is written as:

$$\Omega = 2\phi\sigma_g^2 + I\sigma_e^2$$

where ϕ is the kinship matrix, and I is an identity matrix. To test for evidence of major genes (QTLs), the component of QTL is introduced in the model and the expected covariance matrix for a pedigree is written as:

$$\Omega = \Pi\sigma_a^2 + 2\phi\sigma_g^2 + I\sigma_e^2$$

where Π is the matrix whose elements ($\Pi\sigma_m^2\pi_{jl}$) provides the predicted proportion of a gene that individual j and l share identity by descent (IBD) at a QTL linked to a genetic marker locus. Marker-specific IBD matrixes (Π_m) were generated independently for all the 344 markers across the genome. Multipoint IBD matrixes were then generated at 1-cM resolution by incorporating the IBD matrixes at all the neighboring markers and mapping distances between these markers. To test for linkage, the likelihoods of the two models (with the variance due to the QTL estimated or fixed to zero) is compared. Twice the difference in loge likelihood of these two models yields a test statistics that is asymptotically distributed as a $\frac{1}{2}:\frac{1}{2}$ mixture of a ${\chi_1}^2$ variable and a point mass at zero because the estimated variance due to QTL was fixed to a boundary in the nested model.[20] The difference between the two $\log_{10}$ likelihoods produces a LOD score that is equivalent to the classical LOD score of linkage. Tests for linkage and its effect are repeated throughout the genome.

Table 1 Subject characteristics of Dutch families with asthma

Characteristic	Proband (n=200)	Spouses (n=200)	First-degree offspring (n=530)
M:F	124:76	76:124	237:293
Mean age (years) [range]	52.1 [37-76]	51.1 [33-76]	24.0 [6-53]
PC_{20}<32 mg/ml (%) [n]	88.2 [170]	26.1 [199]	46.5 [525]
FEV_1 predicted (%)	69.7	98.4	93.6
FEV_1 < 80% predicted (%)	61.4	9.0	12.1
Reversibility[a] (baseline)	77.7	21.5	31.8
Reversibility[a] (predicted)	62.9	19.0	25.8
Mean IgE (IU/ml)	92.9	26.3	64.1
Positive skin test (%)	81.9	30.6	54.1

[a] Change > 9%

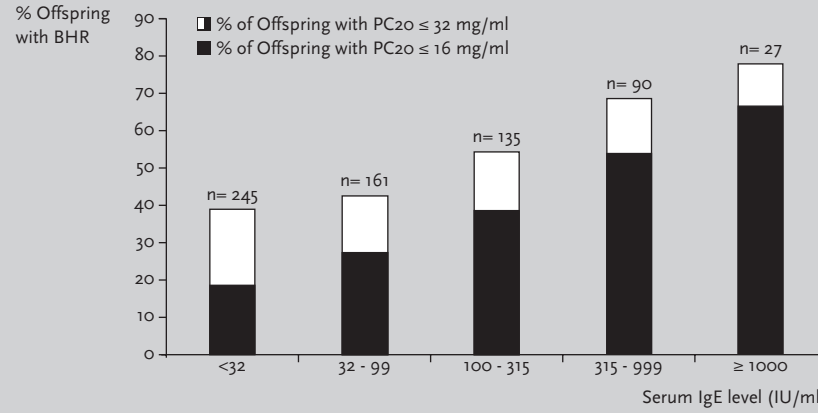
RESULTS

Characteristics of patients

Demographic and clinical characteristics for the family members are shown in table 1. There were more male than female probands; the mean age for the probands was 52 years (51 years of age for spouses and 24 years for children). Although all probands were hyperresponsive at the time of original testing, 12% were not hyperresponsive at current testing (30 were not retested due to low lung function, e.g. FEV_1<40% predicted). A large proportion of probands (82 %) were skin test positive.

Individuals with BHR had significantly higher mean IgE than did individuals without BHR (83 vs. 35 IU/ml, t=9.22, p<0.0001). The relationship between total serum IgE levels and the presence and degree of BHR is shown in figure 1. In addition, skin-test-positive individuals had significantly higher IgE levels than individuals with a negative skin test (112 vs. 26 IU/ml, t= 17.39, p<0.0001). Results from multiple-regression analysis, where $\log_{10}$(IgE) was the dependent variable and BHR, skin test responsiveness, sex, and age (year) were independent variables, also suggested that IgE was independently associated with these clinical and demographic variables. The estimated regression coefficients were $\beta = 0.20$ ($p = 0.0001$) for BHR, $\beta = 0.59$ ($p = 0.0001$) for skin test responsiveness, $\beta = -0.07$ ($p = 0.04$) for gender (male), and $\beta = -0.006$ ($p = 0.0001$) for age.

Figure 1 Relationship between total serum IgE levels and bronchial hyperresponsiveness, in the offspring of 200 asthmatic parents



Correlation coefficients of various relative pairs

Correlation coefficients (r) for various pairs of relatives, with equal weights to all pairs, are reported in table 2. The results with equal weights to pedigrees and to nuclear pedigrees were similar (data not shown). There was evidence for familial aggregation of high total serum IgE levels, which was probably the result of a genetic component. The results should be interpreted with caution, since these families were ascertained through an asthmatic parent, rather than being selected from the general population. There was a higher correlation among parent-offspring and sibling pairs, with r = 0.24 and r = 0.31, respectively, than among more distantly related relative pairs (r = 0.12 for grandparent-grandchildren pairs and r approached zero for avuncular and first cousin pairs). As expected for unrelated individuals, there was no evidence of correlation between the spouses(r = -0.06). The heritability (h^2) was estimated to be 0.48 from parent-offspring pairs and 0.62 from sibling pairs.

Table 2. Correlation Coefficient of log (IgE) among different relative classes (equal weights to pairs)

Relationship	number of Pairs	Correlation Coefficient
1st degree relatives		
Parent-offspring:		
Mother-daughter	374	0,28
Mother-son	315	0,20
Father-daughter	363	0,25
father-son	306	0,23
Overall	1358	0,24
Sibling:		
Sister-sister	195	0,28
Sister-brother	342	0,32
Brother-brother	151	0,33
Overall	688	0,31
2nd degree relatives		
Grandparent-grandchild	272	0,12
Avuncular	559	0,02
3rd degree relatives		
1st Cousins	397	0,03
Unrelated		
Spouses	275	– 0,06

One-locus segregation analyses

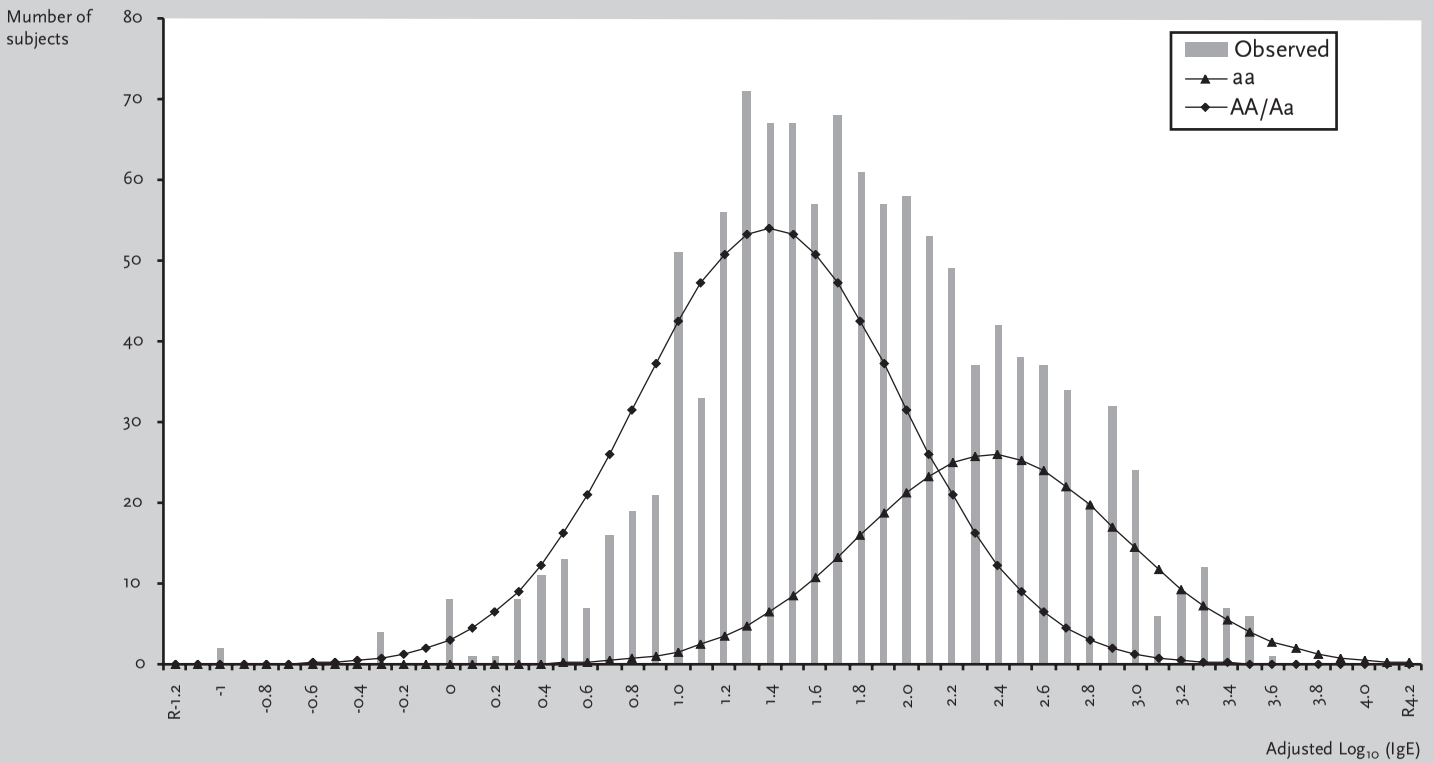
To test whether the familial aggregation of high total IgE levels is due to shared genes or shared environments, complex segregation analyses were performed. In the one-locus segregation analysis, both the sporadic model and mixed environmental model were rejected (table 3). Although Mendelian-only models were rejected, several mixed Mendelian models were not. These models fit the observed distribution of adjusted $\log_{10}$(IgE) levels and were not significantly different from the general model ($p > 0.05$). Of the mixed Mendelian models, the mixed recessive model (a

Table 3. Segregation Analysis of Adjusted log10(IgE) in 200 Dutch Families

		Transmission Probability			Mean									
Model	q_A	AA	Aa	aa	AA	Aa	aa	SD	h2	-2lnL	AIC	chisq	df	P-value
General	0,54	[1]	0,47	[0]	1,63	1,41	2,34	.58	0,57	2356,52	2374,52			
Mix. Env. (Env. + Poly.)	0,55	0,47	= AA	= AA	1,37	1,65	2,15	.65	0,65	2365,95	2379,95	9,43	2	0,009
Mix. Cod. (Cod. + Poly.)	0,55	[1]	[.5]	[0]	1,63	1,41	2,34	.58	0,57	2356,69	2368,69	0,17	3	0,98
Mix. Dom. (Dom. + Poly.)	0,21	[1]	[.5]	[0]	1,49	2,14	= Aa	.63	0,47	2365,90	2375,90	9,38	4	0,052
Mix. Rec. (Rec. + Poly)	0,57	[1]	[.5]	[0]	1,45	= AA	2,32	.58	0,49	2358,42	2368,42	1,90	4	0,754
Cod. Only	0,54	[1]	[.5]	[0]	1,02	1.64	2,42	.5	[0]	2372,41	2382,41	25,89	4	0,00003
Rec. only	0,66	[1]	[.5]	[0]	1,35	= AA	2,22	.55	[0]	2399,15	2407,15	50,63	5	1,02E-10
Polygenic	[1]	n/a	n/a	n/a	1,73	= AA	= AA	.71	0,55	2366,55	2372,55	10,03	6	0,04
Sporadic	[1]	n/a	n/a	n/a	1,74	= AA	= AA	.7	[0]	2487,04	2491,04	130,52	7	0

Note: Env = Environment, Poly = Polygene, Cod = Codominant, Dom = Dominant, Rec = Recessive.

Figure 2. Distributions of adjusted log10(IgE) in 200 Dutch families.



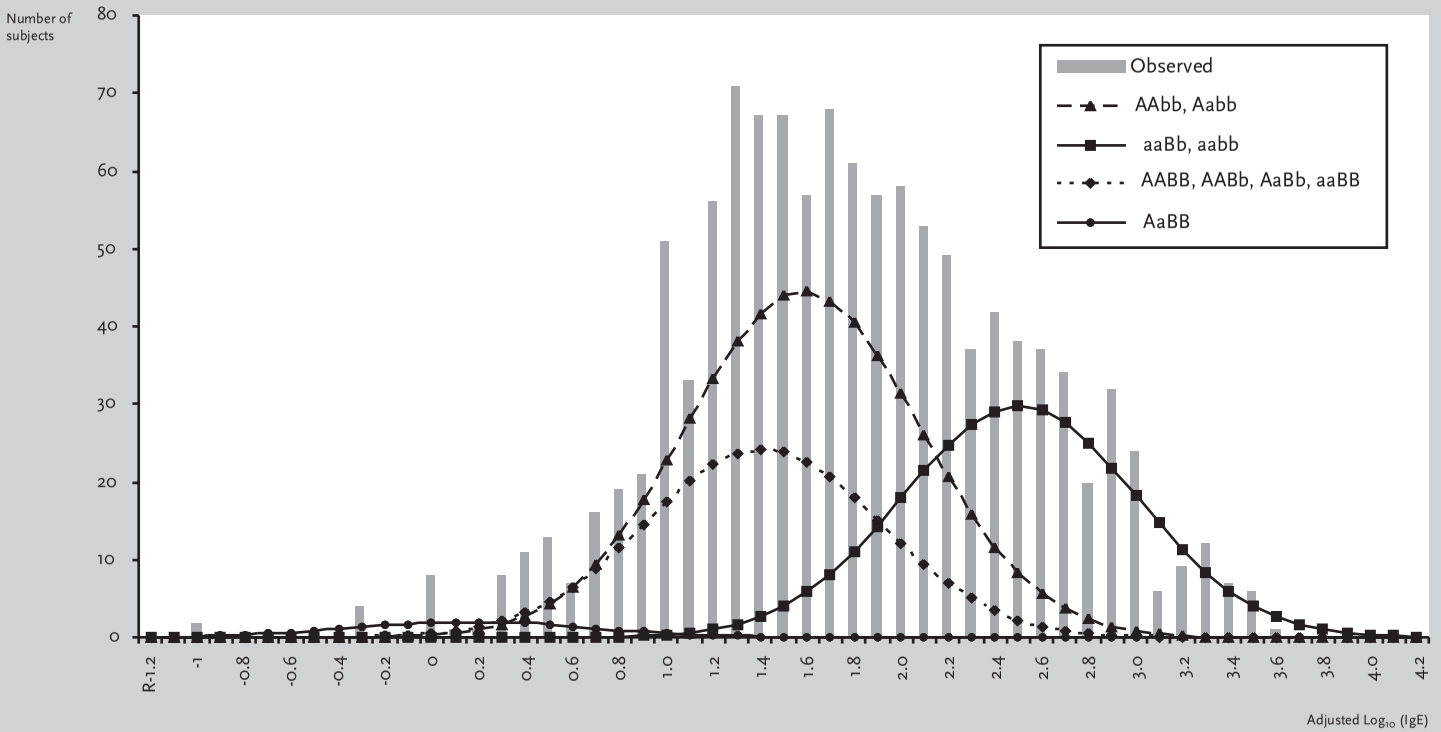
Vertical bar represents the distribution of the observed adjusted log10(IgE).
The two line curves represent the distributions of the three genotypes under the best fitting mixed recessive model.

Table 4. Segregation analysis of adjusted log10(IgE) assuming one- and two-locus models in 200 Dutch Families

	q_a	q_b	Mean for each genotype					SD	h2	-2lnL	AIC
			Locus B		Locus A						
				AA	Aa	aa					
One-locus											
Mixed codominant	0,55			1,63	1,41	2,34		0,58	0,57	2356,69	2368,69
Mixed recessive	0,57			1,45	= AA	2,32		0,58	0,49	2358,42	2368,42
Two-locus											
Two major gene model 1	0,57	0,83						0,51	0,68	2329,19	2355,19
locus A codominant			BB	1,51	0,03	1,43					
locus B codominant			Bb	1,58	1,44	2,59					
			bb	1,53	1,37	2,33					
Two major gene model 2	0,55	0,8						0,5	0,52	2331,25	2347,25
locus A recessive			BB	1,36	0,14	1,36					
locus B dorminant			Bb	1,36	1,36	2,45					
			bb	1,53	1,53	2,45					
Two major environmental risk factor model	0,58	0,83						0,53	0,9	2349,98	2371,98
			BB	1,37	0,98	1,37					
			Bb	1,37	1,37	2,57					
			bb	1,97	1,37	2,22					

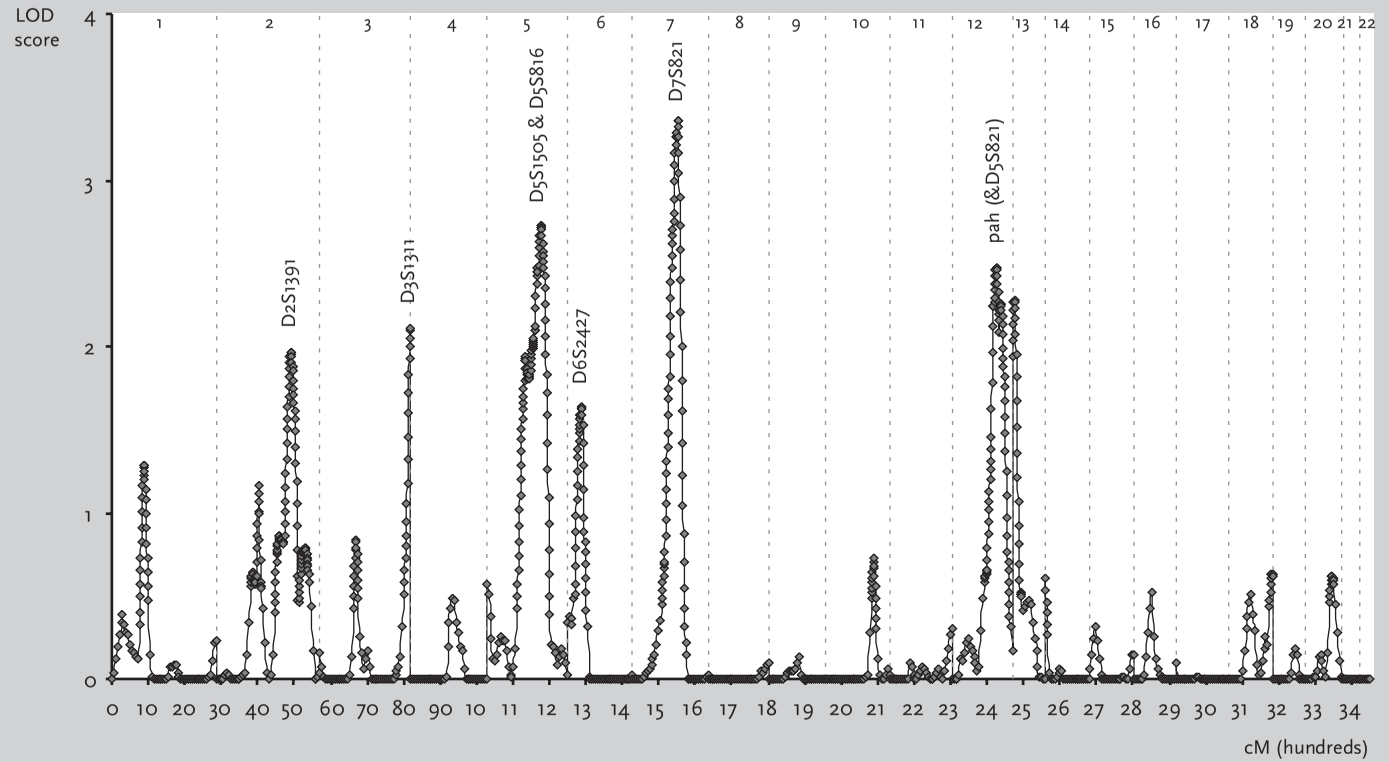
major recessive gene and residual genetic effects) was the best fitting (p = 0.75) and the most parsimonious (smallest AIC) model, although it was only slightly better than the mixed additive model. The MLE of the gene frequency q_a (corresponding to a high IgE level) under the mixed recessive model was 0.57, which results in genotype frequencies of 0.68 for AA/Aa, and 0.32 for genotype aa. When we used GPEs, this recessive gene had a large effect on IgE levels and was responsible for 32.4% of the adjusted log10(IgE) levels in these families. The mean IgE level for genotype aa (μaa) was estimated to be 209 IU/ml, significantly higher than the mean (29

Figure 3. Distributions of adjusted $\log_{10}$(IgE) in 200 Dutch families.



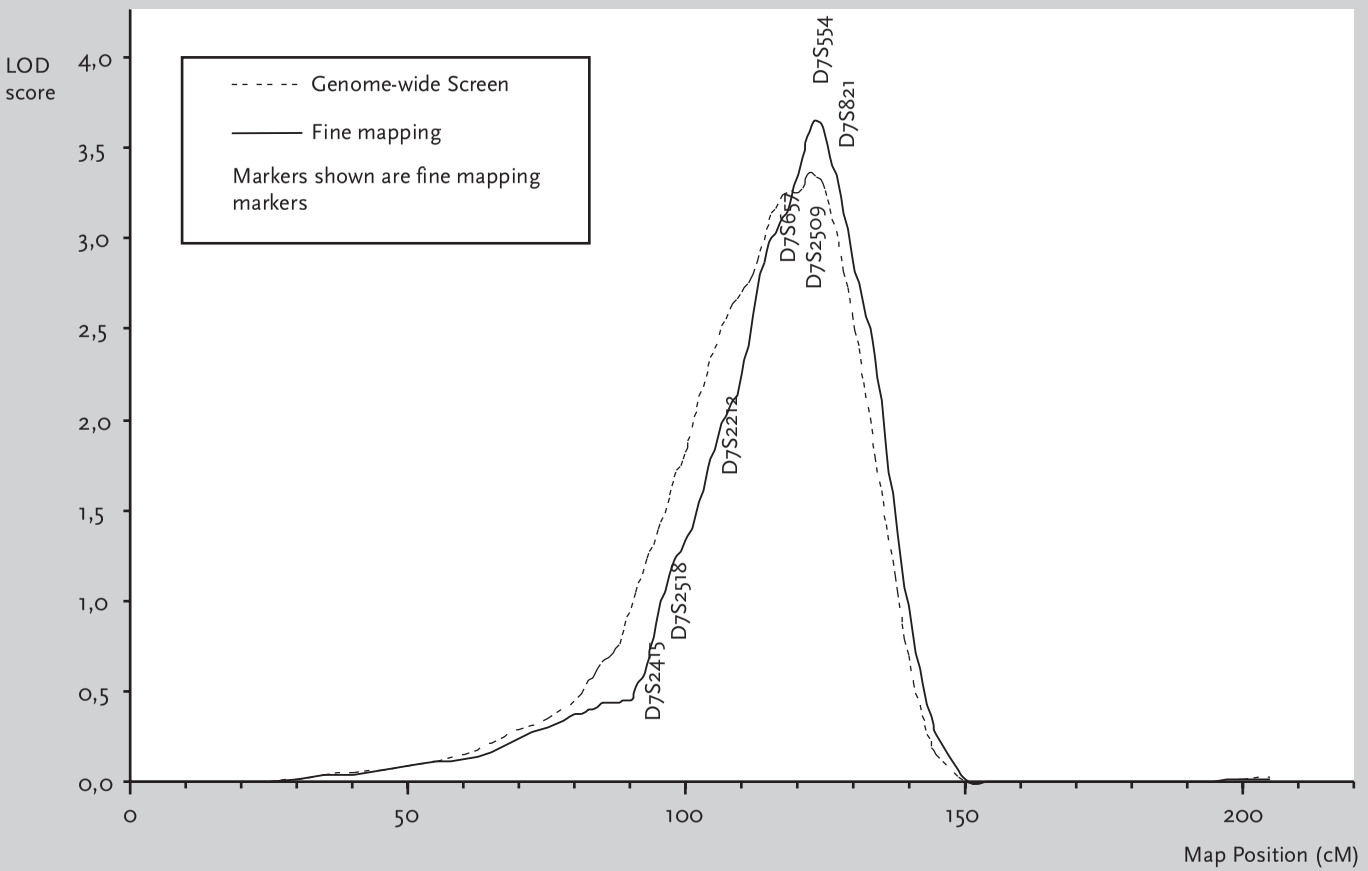
Vertical bar represents the distribution of the observed adjusted $\log_{10}$(IgE).
The four line curves represent the distributions of the nine genotypes under the best fitting mixed two major gene model.

Figure 4. Genome-wide screen for major genes regulating adjusted log10(IgE) levels in 200 Dutch families using variance componant analysis.



Three hundred and forty-four evenly spaced autosomal markers were genotyped. Vertical dotted lines divided the genome into 22 chromosmes.

Figure 5. Multipoint linkage analysis of Log_{10}(IgE) on chromosome 7 using variance component approach



IU/ml) for the other genotypes (μAA, μAa) (t-statistic = 23.89, p = 6.2E-10^9). This model is plotted in figure 2, illustrating the fit of the model. The residual polygenic component was large, which was evidenced by a high heritability estimate (MLE of h^2 = 0.49, 95% C1: 0.35-0.63) and a significant improvement in model fitting compared with a recessive only model (χ^2 = 40.73, df = 1, p = 1.7 x 10^{-6}).

Two locus segregation analysis

When two-locus segregation analysis was performed, the mixed two-major-gene models (two major genes and residual genetic effects) fit the data significantly better than did the one-major-gene models (table 4). Of the several mixed two-major-gene models that were tested, a model with a major recessive gene and a dominant modifier gene was the most parsimonious model. This model (two-major gene model 2) fit the data significantly better than did the one-major-recessive-gene model (χ^2 = 27.17, df = 3, p = 5.42 x 10^{-6}). It also had a much lower AIC value (2347.25) than did the two-major-environmental-risk-factor model (AIC = 2371.98). According to this model, the MLE, gene frequency for the first recessive gene (q_a) was 0.55, similar to the gene frequency of the mixed recessive model under one-locus segregation analysis. The MLE of the gene frequency for the second dominant gene (q_b) was 0.8. The second gene modifies the effect of the first recessive gene so that some of the individuals who were homozygous for the high-risk allele at the first gene did not have high IgE levels. Individuals with genotype aaBB (homozygous high-risk allele at the first gene and homozygous low-risk allele at the second locus) had normal IgE levels (mean = 23 IU/ml) and only individuals with genotypes aaBb and aabb (29.6% of the total sample) had high IgE levels (mean = 282 IU/ml) (figure 3). The two major genes were responsible for 51.3% the adjusted $\log_{10}$(IgE) variance. The first gene was responsible for 40.6% and the second gene was responsible for 9.0% of the adjusted $\log_{10}$(IgE) variance.

Linkage analysis using variance component analysis

Linkage analysis, using the variance components approach, was performed to systematically scan the genome for the locations of the major genes regulating total serum IgE levels. Results of the multipoint linkage analysis for the 344 evenly spaced autosomal markers are presented in figure 4. One chromosomal region with a lod score of 3.36 (p = 0.00004) was observed at 7q and flanked by markers D7S820 and D7S821. This locus explained 39% of adjusted $\log_{10}$(IgE) variance and the residual genetic effect explained 17% of the variance. The evidence for linkage at this region was further supported by two additional analyses. First, the lod score was strengthened by genotyping more markers in the regions. After 7 markers were added to the 30 cM regions, the lod score increased to 3.65 (p = 0.00002) at the same region (figure 5). Second, both the first 92 families and the second 108 families provided a positive lod score at the same region. The lod score at this region was 2.48 (p = 0.0003) for the first 92 families and 1.88 (p = 0.002) for the second 108 families.

To examine the impact that outliers had on the results, the linkage data were reanalyzed for all the chromosome 7 markers after deletion of the six outliers with the highest $\log_{10}$(IgE) values. The lod score changed minimally from 3.65 to 3.74, suggesting that the results were stable and not driven by the few outliers.

Evidence for linkage was at two other regions was also observed. A peak lod score of 2.73 (p = 0.0002) was found at 5q31, flanked by markers D5S666 and D5S402, a result that was consistent with previous findings.[9] This locus explained 37% of adjusted $\log_{10}$(IgE) variance. A peak lod score of 2.46 (p = 0.0004) was found at 12q, flanked by markers PAH and D12S2070. There were several other regions with lod score of ~2. Some of these regions (3 qter and 13pter) were at the tip of the chromosomes and were supported mainly by one marker. The significance and the interpretation of these regions were uncertain and genotyping more markers in these regions is necessary. Other regions (2q, and 6p) were supported by multiple markers.

Discussion

The family-ascertainment scheme of the current study was appropriate for performing both segregation analysis, and variance component analysis for total serum IgE levels. In this study all the probands were recruited from a well-defined population sample in the northeastern Netherlands, where little immigration occurs. The probands were recruited because of prior diagnosis of asthma 25-35 years ago and were evaluated using a standardized protocol.[10] All the children and grandchildren (≥8 years old) of the probands were studied, regardless of their phenotypic status.

Although many studies have demonstrated heritability of serum IgE levels, the precise mode of genetic control has remained elusive. There have been multiple complex segregation analyses investigating the mode of inheritance of total serum IgE within families. Several previous studies have found evidence for a recessive gene regulating high IgE levels, with different estimates of gene frequencies and mean IgE levels. [9,21,22,23] Evidence for a codominant mode of inheritance was reported by Martinez et al. [24] suggesting that homozygotes and heterozygotes have different mean levels although there is clearly overlap between the distributions of total serum IgE levels.

The segregation analysis of the current study represented an extension of the segregation analyses in the first 92 families.[8,9] In the initial study, evidence was found for a mixed recessive gene (a recessive gene and residual genetic effect) and a mixed model with two major genes (two major genes and residual genetic effect) regulating total IgE levels, for a one-locus model and two-locus model, respectively. After including an additional 108 families, we obtained increased evidence supporting these models. Under a one-locus segregation analysis in the total sample of 200 families, the mixed recessive model fit the data as well as the general model (χ_3^2= 1.90, p = 0.59). This could be compared with the relatively poor fit of the mixed

recessive model for the first 92 families (χ_3^2 = 6.90, p = 0.07). [9] In the total sample, the mixed two-major-gene model fit the data significantly better than did the mixed one-major gene model (χ_3^2 = 27.17, p = 5.4 x 10-6), whereas, in the first 92 families, there was a marginal improvement of the best two-major gene model over one-major gene model, (χ_5^2 = 11.9, p = 0.04).

For our previous linkage analyses, we had used a candidate gene approach and had found evidence for linkage to chromosome 5q in the first 92 families. [8,9] We have now completed a genomewide search approach in 200 Dutch families with asthma, using a new analytic method appropriate for investigation of quantitative traits. A region on chromosome 7q reached the criteria for a genomewide significant linkage and provided the strongest evidence for linkage, with a peak lod score of 3.65, p = 0.00002.[25] Linkage-analysis results also identified two regions with evidence for linkage at 5q and 12q, both regions have been reported elsewhere and are rich with appropriate candidate genes for immunologic and allergic responses.

There were two linkage reports that systematically searched across the genome for the loci regulating total serum IgE. In a linkage study of 364 subjects in 80 nuclear families subselected from a population sample of 230 families in Busselton in Western Australia, Daniels et al.[26] used 20cM intervals for their genome screen and, after genotyping 274 autosomal markers, identified four regions that are likely to contain the genes regulating total serum IgE levels; the four regions were 6p, 7p, 11q13, and 16q2. In another genomewide screen linkage study, carried out in Germany in a smaller sample (97 families), Wjst et al[27] reported four regions (2p, 6p, 9q2, and 12q) linked to loci regulating total serum IgE.

The possible explanations for the varying results from the various studies include the differences in study populations, ascertainment schemes, sample sizes, and analytical methodology. The assumptions required for some of the analytical methods oversimplify the complexity of the trait and are probably inappropriate for modeling the genetic regulation of total serum IgE levels. This is especially true for segregation analysis where most of the approaches assume only one major factor (a gene). Obviously, this is not realistic for a common trait such as high total serum IgE levels. Alternatively, segregation analysis assuming two major genes, although probably still too simple a model, may significantly improve our ability to model the trait. The effects of two genes and their interactions can be explored under the two-locus model.

There have been difficulties in linkage analysis of quantitative traits in humans. Parametric linkage analysis of quantitative traits has been rarely used, because it requires specifying a genetic model to describe the mode of inheritance, which is usually uncertain. Furthermore, when the SD within the genotype is too large and the distribution of the trait overlaps among the genotypes, the parametric approach is usually uninformative. In some of the earlier studies, non-parametric analysis was performed using the phenotypic information of sib-pairs only, either because other relatives

were not characterized or the all relative pair approach was not yet available for quantitative traits. Recently, an alternative quantitative trait linkage analysis, a variance-component method, was developed.[28-31] The variance-component method allows for marker-specific effects, residual additive genetic effects, and random environmental effects. The variance component approach for a linkage study of a quantitative trait has a number of compelling features.[32,33] It uses all of the available inheritance information in a pedigree of any size or structure. This not only avoids the violation of true bivariate structure of a sib-pair, which can occur in analyses of the phenotypic sib-pair difference, but also uses the available information more efficiently and therefore can achieve greater power to detect linkage. Second, the variance-component approach resolves the problem of independence of sib-pairs within a family by maximizing the likelihood of a pedigree that is jointly conditional on all members of the pedigree.[34] The violation of independence of sib-pairs has been a significant problem, because different weighting schemes have tended to produce strikingly different results.[35]

The results of the present study can be summarized as follows. First, the results confirm the strong association between total serum IgE levels and bronchial hyperresponsiveness and allergy. Second, as observed in previous studies, there is strong aggregation of total serum IgE levels within families. Third, the segregation analysis provides evidence that major genes with residual genetic effects are responsible for the aggregation of total serum IgE levels. The presence of at least two major genes, one behaving as a recessive gene and another behaving as a dominant modifier gene, is consistent with the observed distribution of IgE levels in these families. Finally, a genomewide search using variance-component approaches identified several regions that are likely to contain the major genes regulating total serum IgE levels. Regions on chromosomes 5q, 12q, and 6p have been reported elsewhere.[8,9,21,26,27,36,37] The novel region on chromosome 7q was confirmed by typing additional markers and needs to be replicated in other populations.

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Electronic-Database Information

Accession number and URLs for data in this article are as follow:
Online Mendelian Inheritance in Man (OMIM),

http://www.ncbi.nlm.nih.gov/Omim (for IgE [MIM 147061] and asthma [MIM 600807])
LINKAGE: ftp://linkage.rockefeller.edu/software/linkage
PAP: Pedigree Analysis Package,
ftp://ftp.genetics.utah.edu/pub/software/pap
S.A.G.E. Statistical Analysis for Genetic Epidemiology,
http://darwin.cwru.edu/pub/sage.html
SOLAR: Sequential Oligogenic Linkage Analysis Routines,
http://www.sfbr.org/sfbr/public/software/solar/index.html

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Chapter 6 | Genome-wide search for atopy susceptibility genes in Dutch families with asthma

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Abstract

Background

Atopy is a phenotype associated with asthma that has a heritable component. However, the exact role of atopy susceptibility genes is unclear.

Objective

To study the familial aggregation and association of atopic phenotypes within family members of patients with asthma and to identify chromosomal regions that may contain genes that regulate different atopic phenotypes.

Methods

Genome-wide screen and linkage analysis for atopic phenotypes were performed in 200 families (n=1174) ascertained through a proband with asthma. Specific IgE to common aeroallergens (Phadiatop assay) and Der P1, skin tests (positive to house dust mite or ≥1 out of 16 allergens) and peripheral blood eosinophils were evaluated and compared to the linkage results for total serum IgE.

Results

There was a clear familial aggregation of atopy. A high total serum IgE level in combination with a positive Phadiatop, or a normal total IgE level in combination with a negative Phadiatop was found in 56.1 % of the probands and 66.9 % of the offspring. Several chromosomal regions showed evidence for linkage to an atopic phenotype: chromosomes 2q, 6p, 7q, and 13q, and also showed evidence of linkage with total serum IgE (Xu et al., 2000). Specific regions of interest for atopic traits were also detected on chromosomes 11q, 17q, and 22q.

Conclusions

Atopic phenotypes show familial aggregation. Total IgE and specific IgE did not show complete overlap in members of families ascertained through a parent with asthma. Specific chromosomal regions appear to be important in susceptibility to different phenotypes of atopic responsiveness.

Introduction

The prevalence of atopic diseases that include asthma and other allergic disorders has been rising throughout the world during the last two decades.[1] Atopic diseases are caused by interactions between environmental factors and host or genetic susceptibility.[2] This worldwide increase in atopic diseases appears to be caused by recent changes in environmental exposures that include indoor allergens, air pollutants as well as alterations in the characteristics of respiratory infections in early life with a reduction in bacterial etiologies facilitating the development of allergic phenotypes (hygiene hypothesis).[3,4] Allergic responsiveness is common in many populations, and genetic susceptibility is probably due to frequent polymorphisms in multiple genes. Although the frequency of these polymorphisms is probably not changing, an increase in environmental exposure would lead to expression of the phenotype in previously unaffected but genetically susceptible individuals.[5] Thus, it is important to identify chromosomal regions and the genes that regulate overall (global) and specific allergic responsiveness. This will permit early identification of individuals at risk for allergic diseases and facilitate preventive public health measures by understanding gene-environment interactions.

Our primary approach to identify genes for atopy is through positional cloning, where regions of the human genome that may contain atopy susceptibility genes are identified by linkage analysis in families.[6] Subsequent fine mapping and positional candidate gene studies will lead to the identification of atopy susceptibility genes. The close interrelation of asthma and atopy has enabled us to study atopic phenotypes in families ascertained through probands with asthma. A genome-wide linkage analysis was performed for five phenotypes related to atopy: specific serum IgE levels and allergy skin tests to common aeroallergens, specific IgE to Der P1, skin test to house dust mite and peripheral blood eosinophils. These results are compared to linkage results of total serum IgE levels, which have been published previously.[7] The aims of the present study are first to study the familial aggregation of atopic phenotypes; second to examine the association of atopic phenotypes within family members of patients with asthma and third, to identify chromosomal regions that may contain genes that regulate different atopic phenotypes. Finally, we will compare these linkage results to previously published linkage studies in allergic disorders.

Methods

Study population

Between 1962 and 1975, patients with asthma from the northern part of the Netherlands were referred to Beatrixoord, a regional asthma center in Haren, the Netherlands. These newly diagnosed patients with symptomatic asthma who were not experiencing a current asthma exacerbation underwent a standardized, complete evaluation. For inclusion in the current study, at the time of initial testing all probands were younger than 45 years of age, displayed characteristic asthma symptoms, and had bronchial hyperresponsiveness (BHR) to histamine ($PC_{20} \leq 32$ mg/ml, 30 seconds inhalation protocol.[8] Two hundred probands were restudied between 1990 and 1999 together with their spouses, a minimum of two children, and when available their children's spouses and grandchildren. The study was approved by the Medical Ethics Committee of the University Hospital Groningen, as well as the Institutional Review Boards of the University of Maryland and Wake Forest University. For adults, written informed consent and for children, written parental consent was obtained from all participants.

Clinical and laboratory evaluation

The following atopic phenotypes were studied: allergy skin tests, total serum IgE, specific IgE to a group of common aeroallergens (Phadiatop assay) and to Der P1 and peripheral blood eosinophils.

Allergy skin testing was performed in adults by intracutaneous tests with 16 common aeroallergens, as well as a positive and negative control. Ten skin prick tests were performed in the children with a positive and a negative control. The following allergens were tested in both adults and children: mixed grass and tree pollens, mixed weeds, house dust mite, dog and cat, a mixture of guinea pig and rabbit, horse and the moulds *Aspergillus Fumigatus* and *Alternaria Alternata*. In adults, additional skin tests included a second mix of grass pollens and mixed tree pollens, storage mite, feather mix, and three moulds (*Cladosporium herbarum, Penicillum notatum, Botrytis cineria*) (ALK, Nieuwegein, the Netherlands). The maximum diameter and the perpendicular diameter of the weal were recorded after 15 minutes. An intracutaneous skin test was considered to be positive if the mean weal diameter was ≥ 5 mm; a skin prick test was considered positive if the mean weal diameter was ≥ 3 mm. The skin tests were not used for further analysis if the negative control gave a positive reaction.

Total IgE was measured by solid phase immunoassay in the first 92 families. The mean of two duplicate tests of IgE was used, and measurements were repeated if the difference between duplicates was > 5%. In the second set of 108 families, serum IgE levels were measured by an enzyme linked fluorescent assay (Mini Vidas, Biomerieux Inc., Marcy, France) The two methods showed high correlations in our laboratory ($r=0.95\text{-}0.99$, $p < 0.01$).

Specific IgE was measured by an in vitro test system (Pharmacia CAP system, Phadiatop FEIA) according to the instructions of the manufacturer

(Pharmacia Diagnostics AB, Uppsala, Sweden). This assay is composed of a mixture of inhalant-allergens and is used as a general assessment of allergic responsiveness. It determinates the presence of specific IgE to the following antigens: house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), timothy grass (*Phleum pratense*), birch (*Betula verrucosa*) and olive pollen (*Olea europea*), two different weeds (*Artemisia vulgaris* and *Parietaria officinalis*), a mould (*Cladosporium herbarum*), and finally cat, dog and horse dander. The test was regarded positive if the fluorescence score of the subject's serum was higher than that of the reference as provided by the manufacturer. In the individuals who tested positive by the Phadiatop assay, specific IgE directed against the major allergen Der P 1 of the house dust mite *D. pteronyssinus* was measured by the Cap method (Pharmacia Diagnostics, Uppsala, Sweden). The lower level of detection was 0.35 kU/l. As a qualitative trait, specific IgE to Der P 1 was regarded to be present if the result of the Der P1 assay was class 2 or higher, which is > 0.7 kU/l. Finally, total peripheral blood eosinophils were counted in a counting chamber.

All participants were asked to discontinue asthma and allergy medication before the clinical testing if possible: specifically inhaled corticosteroids were stopped for 14 days, inhaled long acting beta-mimetics; and oral antihistamines for 48 hours, inhaled short acting beta-mimetics and anticholinergics for 8 hours. Asthma patients had not experienced an asthma exacerbation or require a course of oral prednisone during the 6 weeks prior to the study.

Molecular methods

DNA was isolated from peripheral blood by standard methods (Puregene kit, Gentra Inc., Minneapolis, USA).[7] For the genome-wide screen, the Weber version 8 set of markers (Marshfield Center for Medical Genetics) was used which spans the human genome at an average interval of ~10 cM, and consists of 366 polymorphic autosomal markers.[7] Multiplex polymerase chain reaction (PCR) was performed using fluorescently labeled primers. PCR products were separated on denaturing polyacrylamide gels and the fragments were detected using ABI 377 sequencers (Perkin Elmer Applied Biosystems). The fragments were scanned and scored using ABI software. A modified version of the program Linkage Designer was used to bin the alleles and check inheritance.[9] Genotyping errors, double recombinants, and inheritance inconsistencies were detected using the LINKAGE and CRIMAP software.

Statistical methods

To examine the familial aggregation of skin test positivity and Phadiatop positivity, nuclear families were stratified by 0, 1 and 2 parents with the trait. Three by two contingency tables were generated showing the percentage of first degree offspring with and without the trait using SPSS 10.0 statistical software. To examine the familial aggregation for eosinophil

levels, eosinophil number was log transformed and corrected for age and sex by multiple linear regression analysis. Correlation coefficients of log (eosinophil numbers) among various pairs of relatives were estimated from the sums of squares and from cross products from the pairs, using the computer program FCOR of S.A.G.E. (Statistical Analysis for Genetic Epidemiology). The option of equal weight to pairs is reported. Heritability was estimated from the variance components appraoch and implemented using the SOLAR (Sequential Oligogenic Linkage Analysis Routines) program.[10]

To study the overlap of high total serum IgE, positive allergy skin tests and the presence of serum specific IgE to common aeroallergens, three by three contingency tables were generated. High total serum IgE levels were defined as total serum IgE levels in the highest tertile for adults (≥ 18 years) and children (6 - 17 years). This resulted in cut-off values for adults of IgE ≥ 98 kU/l, and for children of IgE ≥ 190 kU/l. Skin test positivity was defined as the presence of one or more positive skin tests; specific IgE positivity was defined as the presence of a positive Phadiatop assay. These calculations were performed using SPSS 10.0 statistical software.

Linkage analysis for quantitative variables was performed using variance components analysis as implemented in the SOLAR package.[10] First eosinophil number was log transformed to obtain a normal distribution and age and sex were included as covariates. The variance of a quantitative trait can be decomposed into residual genetic effects, random environmental effects, and quantitative trait loci (QTL). To perform a genome wide screen, the fit of the models with or without the effect of QTL in the observed phenotype and marker genotype data was compared. The difference between the $\log_{10}$ likelihoods of the two models produces a LOD score that is equivalent to the classical LOD score of linkage.

Linkage analyses for qualitative traits were performed using the affected relative pairs method as implemented in GENEHUNTER-PLUS.[11,12] The option of non-parametric methods was used, which compared the observed marker allele identical by descent (IBD) among the various affected relative pairs with its expected values under the null hypothesis of no lin kage. Allele sharing LOD scores were calculated based on the statistic 'Z-all' and assigning equal weight to all families using the computer program ASM. [12] All linkage results with a minimal LOD score of 1.0. are reported. These linkage results were compared to the published literature and the Asthma Gene Database (http:// cooke.gsf.de/asthmagen).[13] A replication was defined as evidence for linkage ($p \leq 0.01$) of an asthma or atopy associated trait with the same or a closely linked marker (< 10 cM).

Results

Study population

The clinical characteristics of the probands and their family members included in the genome screen are illustrated in Table 1. Eighty-two percent of the probands with asthma had one or more positive skin tests compared to 31 % in the spouses. Specific IgE as detected by the Phadiatop assay was present in 72 % of the probands and 15 % of the spouses. First-degree offspring showed frequencies intermediate between values of probands and spouses: 53 % had a positive skin test and 44 % a positive Phadiatop.

Familial aggregation of skin test positivity, Phadiatop assay and eosinophil number

Heritability estimates (h^2) were highest for specific IgE to Der P1 (0.57); h^2 was 0.41 for Phadiatop; 0.30 for log(eosinophil count); 0.29 for skin test to house dust mite, and 0.25 for skin test positivity.

The prevalence of skin test positivity was 42.7% in children in the 26 fa milies in which none of the parents had a positive skin test. This prevalence was 52.3 % in 119 families in which one of the parents had a positive skin test, and increased to 62.1 % in 50 families in which both of the pa rents had a positive skin test. Similar results were observed for the familial aggregation of a positive Phadiatop test (figure 1). The lowest prevalence of these traits was observed in families in which none of the parents had a positive Phadiatop test (29 %), which increased to 62 % in children of whom both of the parents expressed this phenotype.

Figure 1 Familial aggregation of specific IgE to aeroallergens

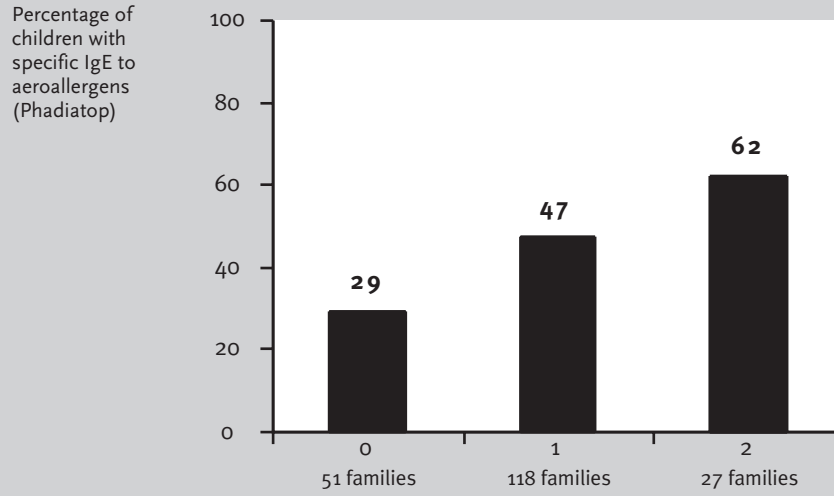
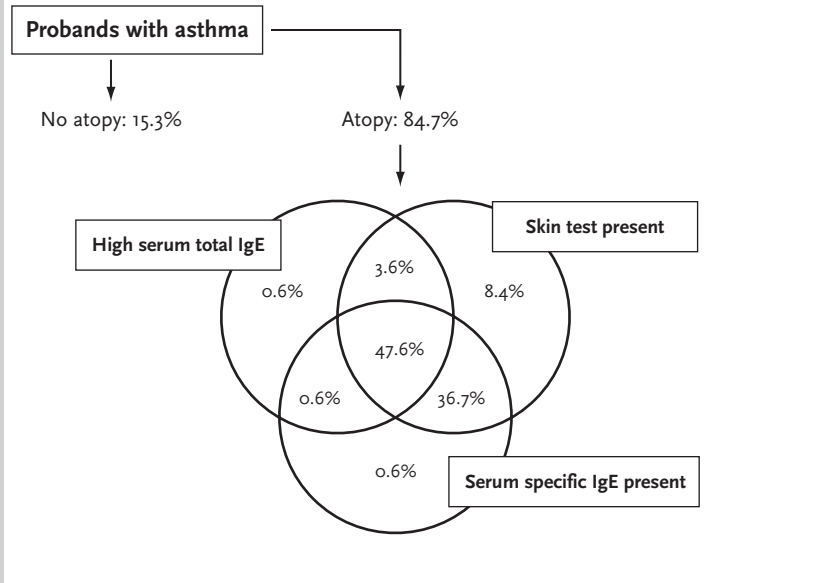


Table 1 Clinical characteristics of atopy in 200 Dutch families from probands with asthma

	Probands (1991-1999)	Spouses		Offspring	
		Of probands	Of 2nd and 3rd degree offspring	1st degree	2nd / 3rd degree
Number	200	201 #	68	531	136
Mean age (SD), years	52.2 (8.5)	51.0 (9.2)	38.8 (6.2)	24.0 (9.0)	12.0 (5.3)
Male, %	62.0	37.8	70.6	45.0	49.3
Total IgE, mean* (range), kU/l	92.0 (1.0 - 2880)	26.2 (0.5 - 1940)	29.1 (3.0 - 1660)	62.8 (0 - 3360)	57.1 (0.5 - 2785)
Positive skin test, %					
≥ 1 Allergen	81.8	31.0	42.6	53.5	31.3
House dust mite	71.4	14.9	19.1	37.6	17.9
Specific IgE, %					
- Phadiatop	72.4	15.1	26.5	44.3	28.8
- Der P1	47.7	3.5	4.4	22.5	10.7
Eosinophils, mean* (range), 10^7/l	9.5 (0 - 126.5)	5.6 (0 - 63.8)	7.9 (2 - 31.9)	10.0 (0 - 294.8)	11.1 (0 - 109)

NOTE. The following clinical data was not present for these reasons: Not enough serum for Phadiatop measurement (23 individuals) or DerP1 measurement (additional 2); Allergy skin test refused by 1 individual; positive response to the negative control (n=4); no blood for total serum IgE analysis (5 individuals) and eosinophil counts (5 individuals). * Mean values for IgE and eosinophils are geometric means. # One proband married twice, both spouses participated in the study.

Figure 2a Overlap of atopic phenotypes in probands with asthma



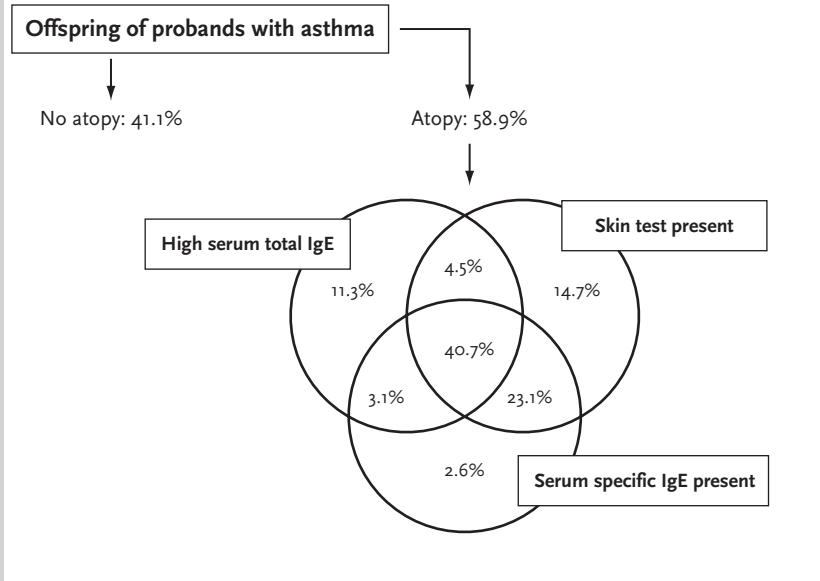
Panel A. Probands with asthma. (n=200) who participated in our genome screen. No atopy defined as normal serum IgE, no skin test positive, and absent serum specific IgE. Atopy defined as high total serum IgE or a positive skin test or specific IgE test. Percentages in Venn diagram is the percentage of all atopic probands with asthma. Missing data on one of three phenotypes in 4 individuals are not included in this figure.

The parent-offspring correlation coefficient of adjusted log(eosinophil) numbers was 0.12 in 1358 pairs (0.14 for 372 mother-daughter pairs, 0.14 for 317 mother-son pairs, 0.10 for 361 father-daughter pairs and 0.07 for 308 father-son pairs). The correlation between siblings was 0.18 in 684 sibling pairs (0.20 between 339 sister-brother pairs, 0.15 for 152 brother-pairs, and 0.16 for 193 sister-pairs). No correlation was observed between grandparents and children (-0.009 in 274 pairs), avuncular pairs (-0.07 in 559 pairs) and cousin pairs (0.002 in 395 cousin pairs).

Overlap of atopic phenotypes in probands with asthma and their offspring

Skin tests and Phadiatop gave consistent results (both positive or both ne gative) in 86.8 % of the probands and 78.6 % of the offspring. However, high serum IgE levels were found in 60 % of subjects who tested Phadiatop positive and in 51% of subjects with one or more positive skin tests. A high total serum IgE level in combination with a positive Phadiatop or a normal total IgE level in combination with a negative Phadiatop were found in 56.1 % of the probands and 66.9 % of the offspring (figure 2, panel A and B).

Figure 2b Overlap of atopic phenotypes in offspring of probands with asthma



Panel B. Offspring (n=667) of probands with asthma who participated in our genome screen. No atopy defined as normal serum IgE, no skin test positive, and absent serum specific IgE. Atopy defined as high total serum IgE or a positive skin test or specific IgE test. Percentages in Venn diagram is the percentage of all atopic offspring of the probands with asthma. Missing data on one of three phenotypes in 21 individuals are not included in this figure.

Genome-wide screen results

Figures 3a to 3e show the results of the genome-wide linkage analysis for Phadiatop, specific IgE to Der P1, skin test positivity, skin test to house dust mite and peripheral blood eosinophils, respectively. In table 2, these linkage results are summarized together with the previously published linkage results for total serum IgE.[7]

Three chromosomal regions showed evidence of linkage for two or more of the five phenotypes in the current analysis: chromosome 11q (skin test to house dust mite, LOD=1.90; Phadiatop, LOD=1.27); chromosome 17q (Phadiatop, LOD=1.38; eosinophils, LOD=1.97; skin test, LOD=1.55; skin test to house dust mite, LOD=1.21), and chromosome 22q (Phadiatop, LOD=1.08; skin test, LOD=1.09; skin test to house dust mite, LOD=1.02).

Four chromosomal regions showed evidence of linkage to one of the atopic phenotypes in the current study and to total serum IgE levels in our previous study: chromosome 2q (eosinophils, LOD=1.49; total serum IgE, LOD=1.96); chromosome 6p (eosinophils, LOD=1.28; total serum IgE, LOD=1.64); chromosome 7q (Phadiatop, LOD=1.04; total serum IgE, LOD=3.36); and chromosome 13q (Skin test, LOD=1.27; total serum IgE, LOD=2.28).

Figure 3a Genome wide search for major genes regulating specific IgE to aeroallergens (Phadiatop)

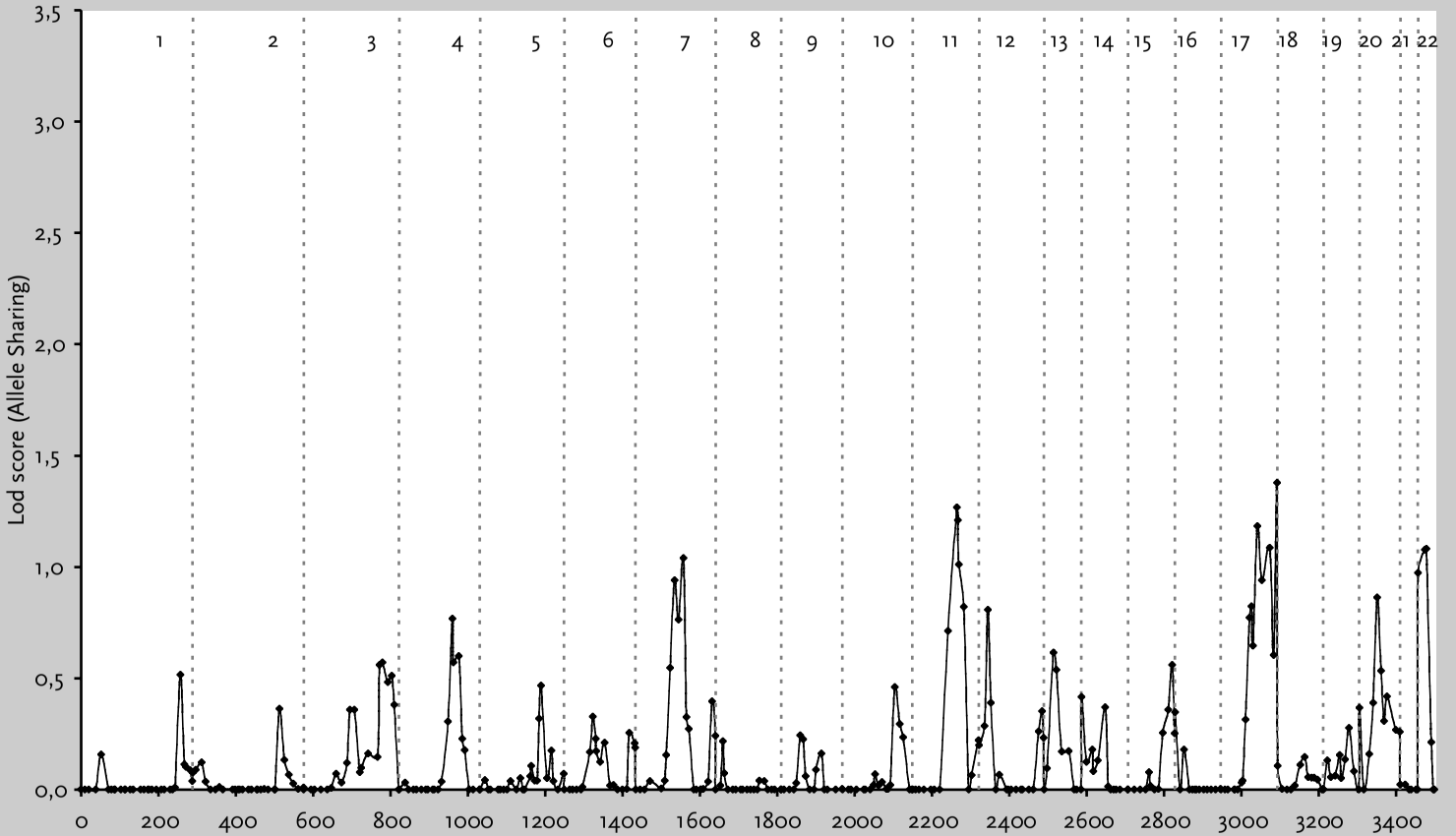


Figure 3b Genome-wide search for major genes regulating specific IgE to Der p 1

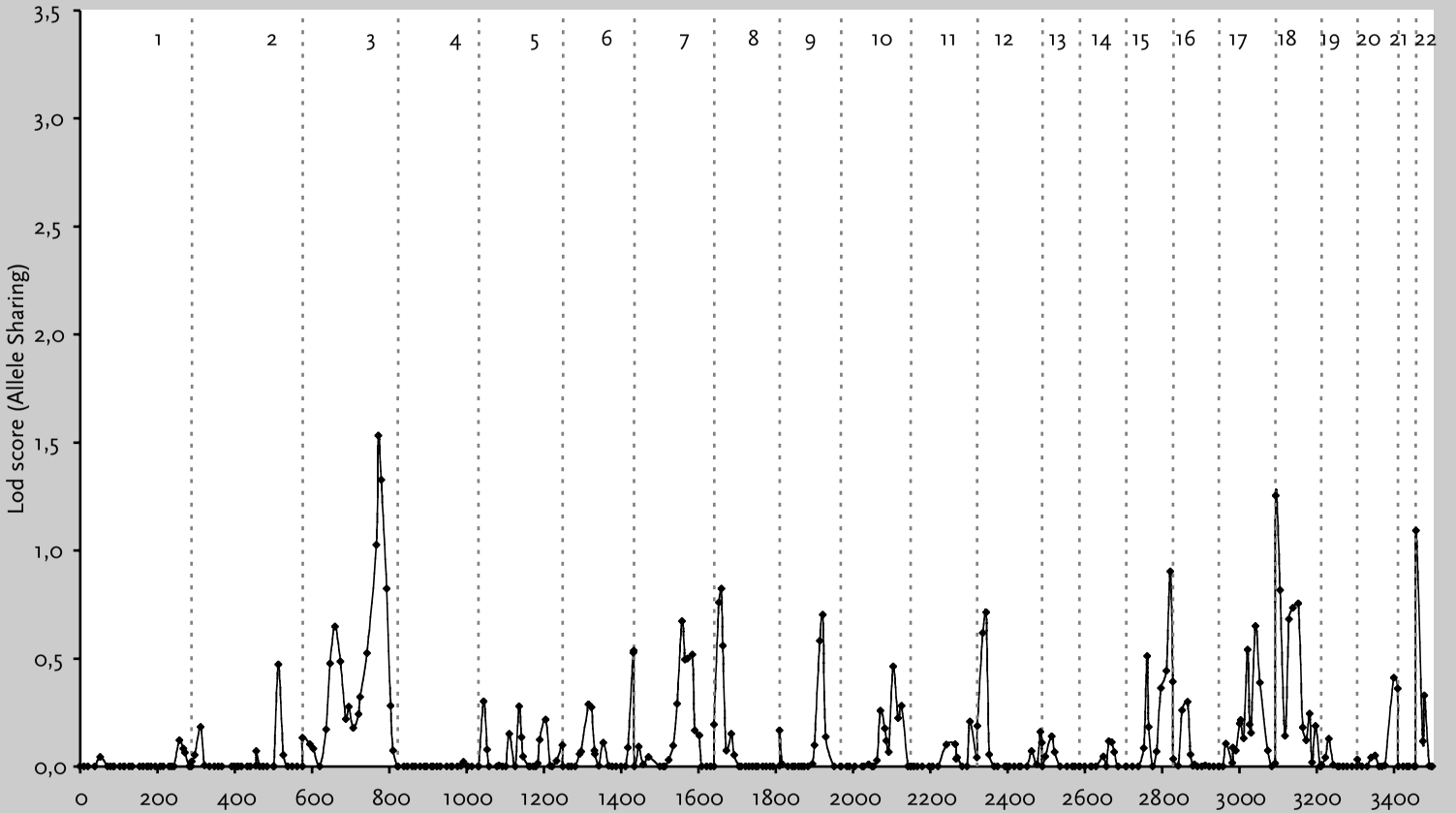
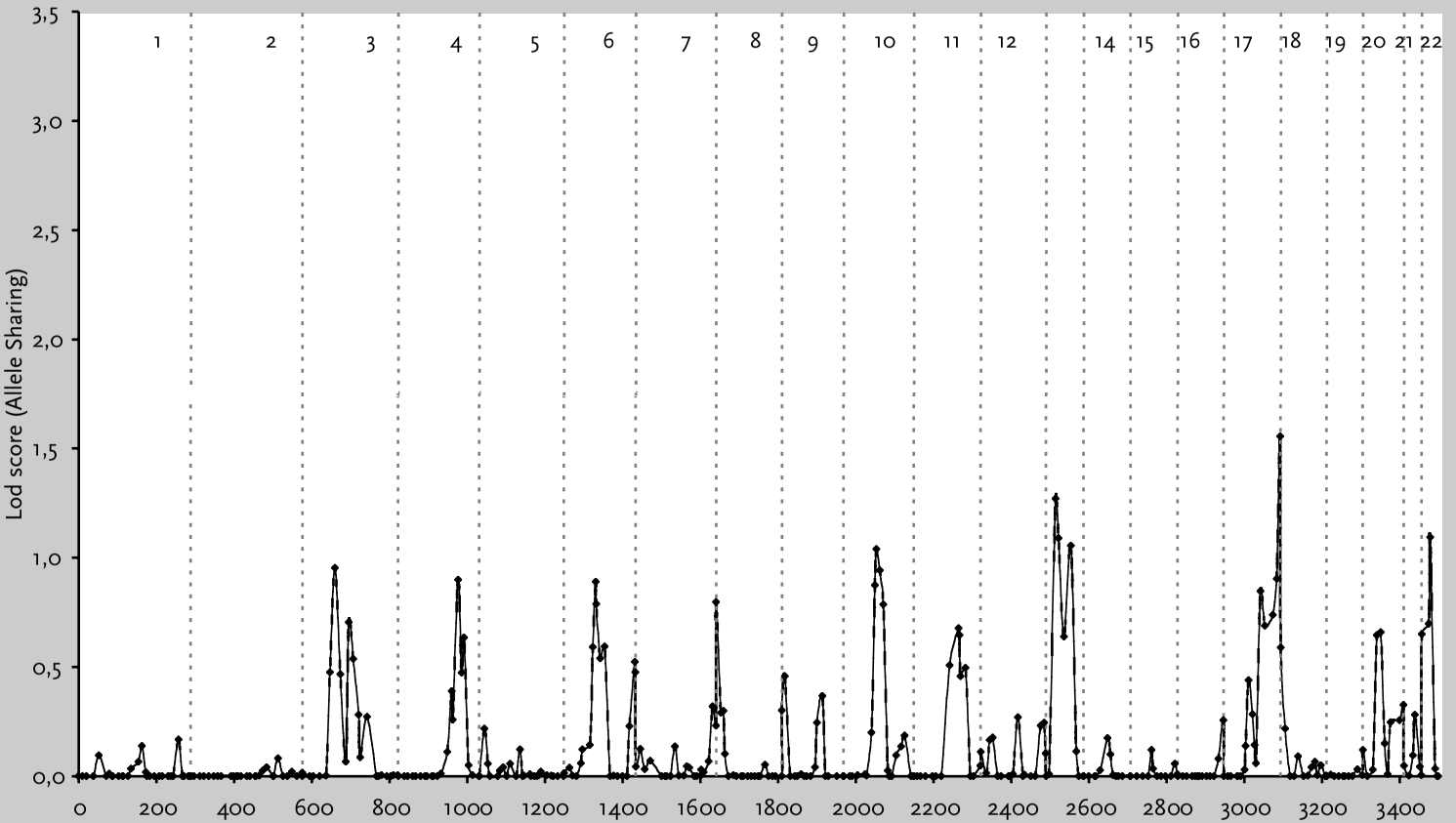


Figure 3c Genome wide search for major genes regulating skin test positivity



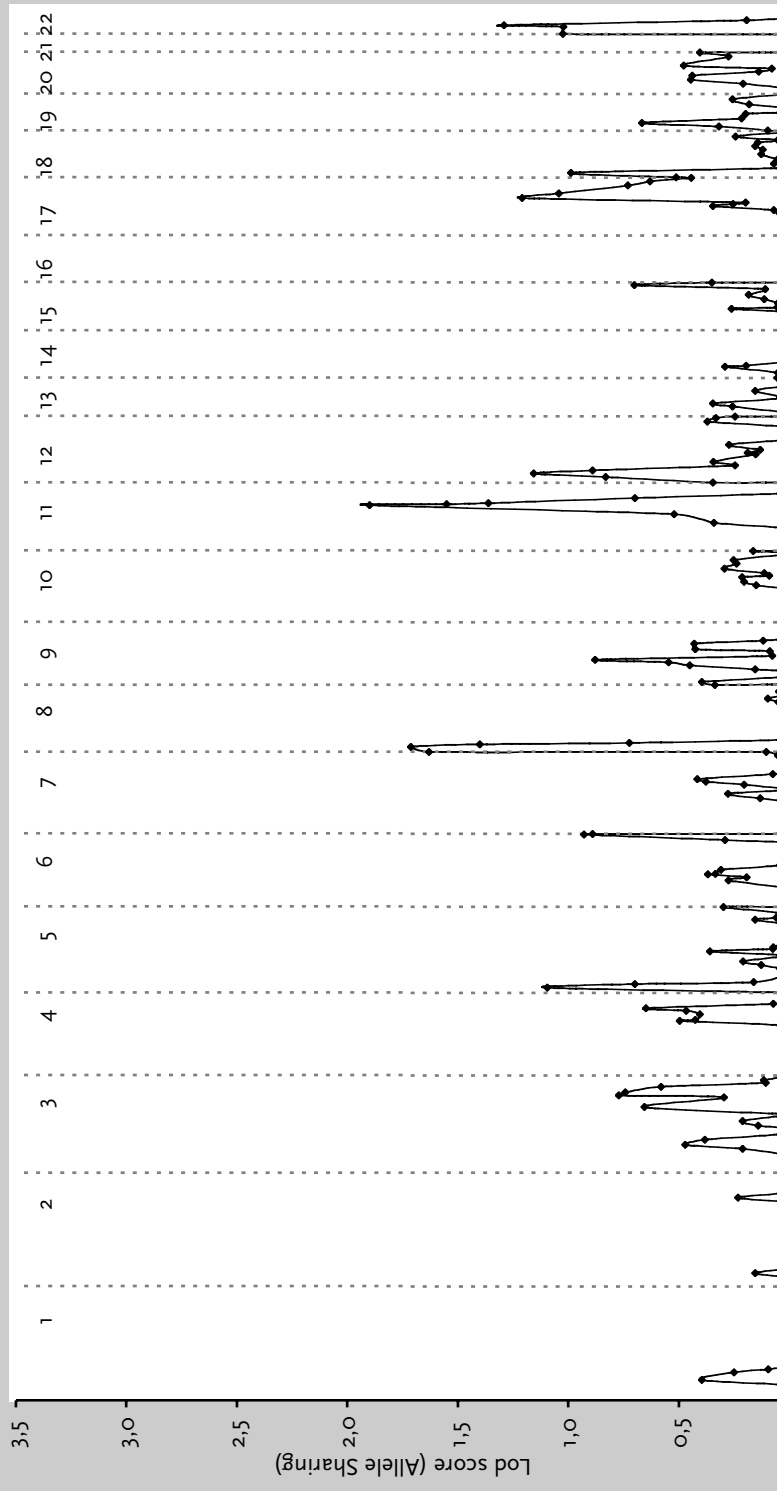


Figure 3d Genome wide search for major genes regulating skin test to house dust mite

Figure 3e Genome-wide search for major genes regulating eosinophils

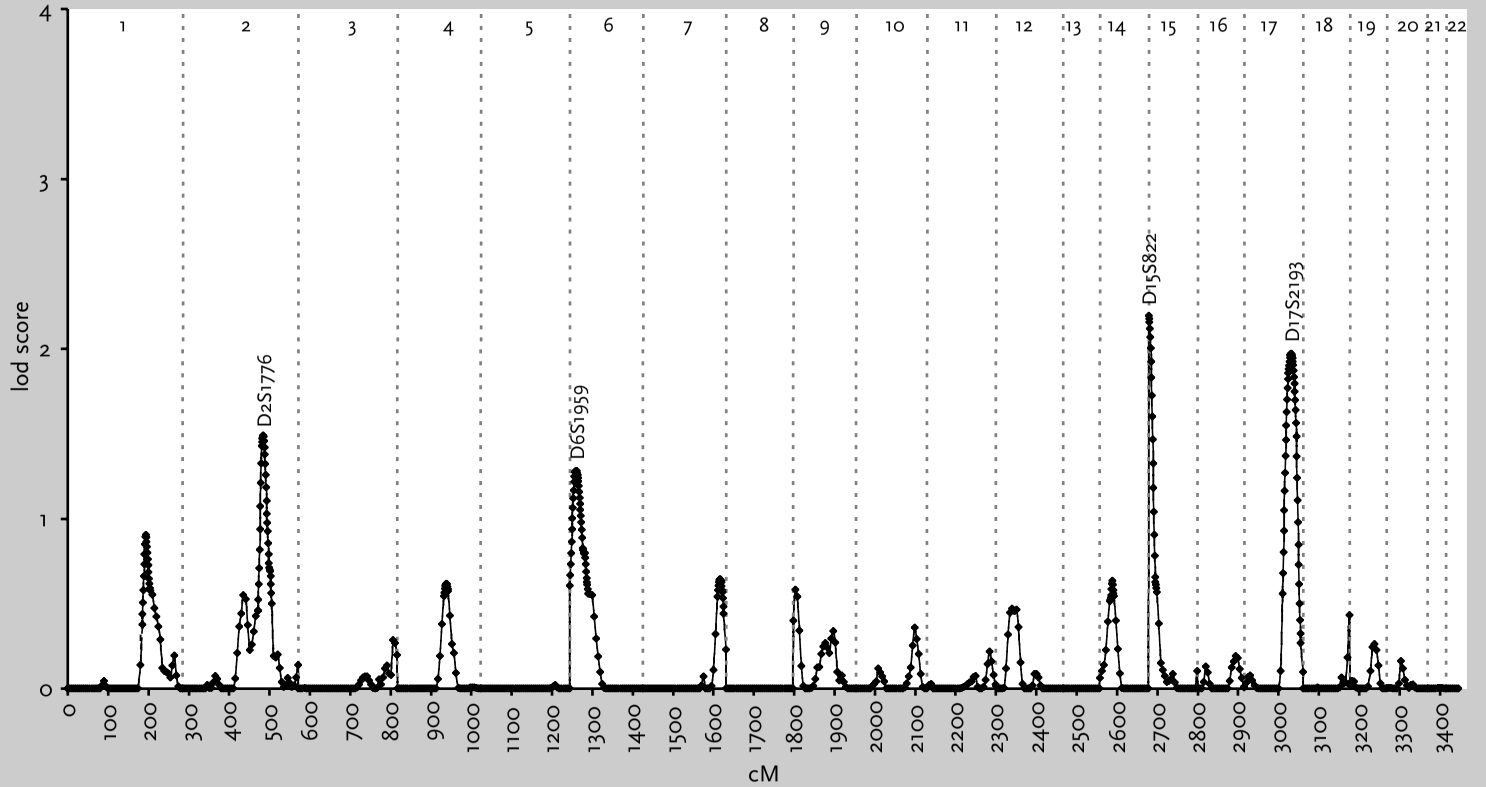


Table 2 Linkage results for specific IgE levels to aeroallergens and to Der P1, skin test positivity, eosinophils, compared to the linkage results of serum total IgE levels

Chromosome	Distance or interval * (sex-averaged in Kosambi cM)	(Flanking) markers	LOD score	Phenotype
1p31	89.49 - 91.89	D1S3728 - D1S2846	1.29	Total serum IgE
2q24-q32	173.00 - 186.21	D2S1776 - D2S1391	1.49, 1.96	Eosinophils, total serum IgE
3q25-q26	181.87	D3S3053	1.53	Specific IgE to Der P 1
3q29	224.88 - qter	D3S1311	2.11	Total serum IgE
5p15	7.77	D5S2849	1.09	Skin test to house dust mite
5q23-q31	129.83 - 139.33	D5S1505 - D5S816	2.73	Total serum IgE
6p24-p22	25.08 - 34.23	D6S2434 - D6S1959	1.28	Eosinophils
6p21	42.27 - 53.81	D6S2439 - D6S2427	1.64	Total serum IgE
7q11-q22	98.44 - 109.12	D7S820 - DS7S821	3.36, 1.04	Total serum IgE, Phadiatop
8p23	8.34	D8S277	1.71	Skin test to house dust mite
10q21-22 §	Est. 85 §	D10s1 §	1.04	Skin test
11q22	101.75	D11S2017	1.21, 1.55	Phadiatop, skin test to house dust mite
12p13	26.23	D12S391	1.16	Skin test to house dust mite
12q23-q24	109.47 - 125.31	PAH -D12S2070	2.46	Total serum IgE
13q12-q13	25.80 - 32.90	D13S1493 - D13S218	2.28	Total serum IgE
13q14	45.55	D13S788	1.27	Skin test
15q11	12.30	D15S822	2.19	Eosinophils
17q21	82.00	D17S1290	1.21	Skin test to house dust mite
17q23	89.32 - 100.02	D17S2193 - D17S1301	1.97	Eosinophils,
17q25	116.86	D17S784	1.38, 1.55	Phadiatop, skin test
18p11	Est. 1.5 #	D18S178 #	1.25	Specific IgE to Der P1
22q11	4.06	D22S420	1.09, 1.02	Specific IgE Der P1, Skin test to house mite
	19.32	D22S345	1.08, 1.09	Phadiatop, skin test

LOD scores over 1.0 are summarized for qualitative traits. The highest LOD scores for quantitative traits are shown. * Map distance taken from Marshfield map. § D10S1 is a not in the Marshfield map. Chromosomal distance was estimated relative to D10S1225(80.77 cM) and D10S1432 (93.92 cM) # D18S178 not in Marshfield map. Chromosomal distance was estimated relative to D18S976 (distance 12.8 cM)

Other chromosomal regions showed evidence of linkage to a single atopic phenotype: chromosome 3q (LOD=1.53) and 18p (LOD=1.25) for specific IgE to Der P1; 10q (LOD=1.04) for skin tests; 5p (LOD=1.09), 8p (LOD=1.71), and 12p (LOD=1.16) for skin test to house dust mite; and 15q (LOD=2.19) for eosinophils.

Discussion

This study shows evidence of familial aggregation of skin test reactivity, elevated specific IgE to aeroallergens and eosinophil number. There are concordant results in the expression of high total IgE and specific IgE as detected by the Phadiatop assay in 56 % of the probands with asthma and 66.9 % of the offspring. In the 200 families ascertained through a proband with asthma, there is preliminary evidence for linkage of atopic phenotypes to multiple chromosomal regions (Table 2). These linkage results for several atopic phenotypes provide an important basis to identify specific genes that regulate host susceptibility for allergic responsiveness.

We have found several chromosomes to be implicated in the expression of atopy. As has been observed in other studies, genome-wide criteria for fin ding highly significant linkage are not always reached.[14] There may be several explanations for this finding. First, although a genetic contribution to atopic traits has been well established, heritability estimates of various atopic phenotypes vary ranging for total IgE from 47 to 74 %.[7,15,16] Estimates of heritability have been reported to be lower for other atopic phenotypes: 34 % for the the RAST index and 35 % for skin test positivity. [15,16] In these family data, variance components analysis confirmed these findings: heritability estimates for total IgE were 55%, Phadiatop 41% , and skin test 25 %. Based on the genetic contribution to the trait, it is therefore plausible that the most significant results for linkage will be obtained for total serum IgE.[7,17] In addition, the study of this quantitative trait (total serum IgE) may have better power to detect linkage than a qualitative trait (specific IgE). Second, it is unknown how many genes contribute to atopy. If each of these genes itself confers a modest increased risk to develop atopy, the trait may be heritable, but the power to find significant linkage produced by one gene in a specific chromosomal region will not be high.[18] We have previously shown using a two-locus segregation analysis on total IgE that two major genes account for 51.3 % of the variance in total serum IgE levels in this population.[7] No estimate of the number of (major) genes for other atopic phenotypes has been published. Third, specific environmental factors may interact with different genes to result in the expression of atopic responses. Thus, a large proportion of the population may carry a susceptibility allele, yet in only a part of these individuals the susceptibility allele is associated with the disease due to the exposure to a specific environmental factor. Fourth, other effects such as a parent of origin effects due to imprinting or gene-gene interactions have not been evaluated in most genet-

Table 3. Comparison of linkage results for atopic traits of the present study with published results (significance level: $p \leq 0.01$)

Chr.	Distance or interval (cM)	Phenotype					
		Asthma & BHR	Total IgE	Specific IgE	Skin tests	Eosinophils	Candidate region studies (phenotype)
1	89.49 – 91.89	French (asthma, BHR)	Dutch				
2	173.00 - 186.21		Dutch		US - Hutterites (house dust mite)	Dutch	
5	129.83 - 139.33	US – Hutterites (strict and symptom group)	Dutch	US – CSGA (Der P1, P2, and crude) Japanese (1) (atopy**)	US - CSGA (dog and cockroach) Hutterites (pollen and cockroach)		US – Amish (total IgE) Japanese (1) (asthma) Dutch (BHR) US – Tucson (eosinophils)
6	25.08 - 34.23 42.27 – 53.81	US – Hutterites (loose) German Japanese (2)	Dutch German	German (≥ 1 of 13) US – CSGA (Der P1)	Hutterites (house dust mite and molds) Australian (atopy*)	Dutch German	
7	98.44 – 109.12	US – Hutterites (BHR) Australian (BHR)	Dutch	Dutch (Phadiatop)	Hutterites (pollen and cockroach)		
8	0.73 – 22.41	US- Hutterites (BHR)			Dutch (house dust mite)		English (house dust mite)
11	100.62 - 105.74			Dutch (Phadiatop)	US - Hutterites (cockroach) Dutch (house dust mite)	Australian	European-HDM (house dust mite)
12	109.47 – 125.31	Japanese (2) Hutterites (loose)	Dutch		Hutterites (molds)	French	English (asthma and total IgE) Barbados (total IgE)
13	25.80 – 45.55	Japanese (2)	Dutch		US - Hutterites (pollen) Dutch (one of 16) Australian (atopy*)	French	
17	82.00 -116.86	US – Hutterites (BHR)	Dutch (Phadiatop)		Dutch (house dust mite) Dutch (one of 16) French (one of 11) US – Hutterites (house dust mite)	Dutch French	

All studies with evidence for linkage ($p \leq 0.01$ for primary phenotype) in the same chromosomal region (spanning 20 cM from peak or interval markers) are included in the table. If multiple markers showed evidence for linkage, the marker with the lowest p-value was included in this table. BHR Bronchial hyperresponsiveness. Chr. Chromosome. Map distance taken from Marshfield map. Data were taken from original papers and the Asthma Gene Database. [13]

Sources and definitions of the phenotype

- Australian. Atopy* was defined as one of the three (high serum total IgE level, one skin test postive, or one specific IgE positive). Reference: Daniels *et al*. [22]
- Barbados. Reference Barnes*et al*. [38]
- Dutch: BHR to histamine. References: Xu *et al* [7], Postma*et al*. [31], present study
- English. Asthma defined as positive answer to the question; have you ever had asthma? Reference Wilkinson *et al*. [15]
- European - HDM. Families from England, Germany, Portugal, and Italy. Subpopulation English families is part of this study. Reference. Kurz *et al*. [34]
- European - AD (atopic dermatitis). Families from Germany, Italy, Sweden, and Netherlands. Reference Lee *et al.* [25]
- French - BHR to methacholine. Asthma defined as a the presence of asthma attacks or attacks of breathlessness at rest with wheezing, combined with one of the following four (BHR, reversibility >12 % after use of a bronchodilator, ever hospitalisation for asthma, or asthma therapy. Reference: Dizier *et al*. [21]
- German. Asthma: History of physician diagnosed asthma. References: Wjst *et al*. [17;24]
- Japanese. (1) Asthma is defined as the presence of recurrent episodes of wheezing and shortness of breath in the preceding year, that is reversible. Atopy ** was defined as the presence of one of three (high total serum IgE levels or the presence of specific IgE to aeroallergens. Reference: Noguchi *et al*. [39]
  Japanese (2) Asthma defined as the presence of two or more episodes of wheezing and shortness of breath, whish was reversible and the presence of specific IgE to D. farinae. Reference: Yokouchi *et al*. [26]
- US-Hutterites. BHR to methacholine. Strict asthma: BHR and symptoms. Loose asthma: BHR or symptoms. Symptom: asthma symptoms. Reference Ober*et al*. [20]
- US - CSGA. Asthma defined as presence of two of three symptoms (cough, wheeze, dyspnea), and BHR to methacholine or reversible airways obstruction (≥ 15% increase from baseline). References: CSGA [37], Hizawa *et al*. [23]
- US - Tucson. Reference: Martinez *et al*. [40]
- US - Amish. Reference: Marsh *et al*. [41]

ic studies.[19] An important issue for the correct interpretation of linkage results in genome-wide screens is the level of significance. We reported all results of LOD > 1, since a comprehensive approach showing all linkage results permits meaningful comparisons between different studies. Consistent replication of linkage results in different populations may provide useful evidence for the locations of atopy susceptibility genes. For example, the results of this study suggests a possible role of chromosome 17q for different phenotypes related to atopy (eosinophils, skin test to house dust mite and Phadiatop). Linkage of this region for the same phenotypes was also observed in the US Hutterite and French population (table 3).[20,21] Thus, this region of chromosome 17q represents a replicated regions that requires additional fine mapping to identify potential susceptibility genes for atopy.

Genome-wide screens of atopy related phenotypes have been performed in multiple populations[17,20-26], although families were ascertained using different methods, i.e. through two children with asthma[21,24,26,27], two children with atopic dermatitis[25], a random population sample, a single large pedigree from a genetically homogeneous population of the Hutterites[20], or a child or parent with asthma.[7,22] The type of ascertainment may affect the linkage results (table 4). For example, in the Japanese study, 100 % of the population with asthma were house dust mite sensitive, whereas only 52.1 % of the Hutterites with asthma had one or more positive skin prick tests. Thus, the linkage results found in the Japanese study could be accounted for by genes important in asthma, atopy or both. Replications of similar chromosomal regions with different phenotypes may identify a pleiotropic gene (a gene causing different phenotypes depending on other genetic and or environmental factors). For atopy, evidence for shared and specific genetic factors in the regulation of serum total IgE, bronchial hyperresponsiveness to methacholine and specific IgE to aeroallergens has been suggested.[16] Evidence for shared genetic factors in skin test positivity and bronchial hyperresponsiveness to hypertonic saline has also recently been reported.[28] In addition, the combination of different closely linked genes could account for the linkage signal observed in several chromosomal regions such as 5q, 11q, and 12q. When reviewing all published genome wide screens, a clustering of linkage signals for asthma and atopy can be observed. We speculate that different genes regulate total and specific serum IgE levels. In addition, the association of skin test positivity and the presence of specific IgE with asthma and allergic rhinitis may result from different combinations of 'atopy' and 'asthma' genes.[29,30]

Based on the analysis of five atopic phenotypes in the current study, replicated evidence for linkage to atopy was observed on chromosomes 2q, 6p, 7q, 11q, 13q, and 17q. Evidence for linkage to chromosome 8 was also observed, consistent with previous reports for linkage to asthma. In addition, our previous linkage results for total serum IgE levels are consistent with

Table 4. Characteristics of published genome-wide studies compared to present study

First author	Study population	Number of families (number of individuals)	Ascertainment strategy	Average marker spacing	Asthma or BHR	Atopic dermatitis	Total IgE	Specific IgE	Skin tests	Eosinophils
Daniels [22]	Australian	80 (n=364)	Young families selected from population sample of 230 families	~11.5 cM	X		X		X	X
CSGA [23;37]	US - Caucasian, African American and Hispanics	Asthma: 199 nuclear and 67 extended families. (37) Specific IgE to Der P: 45 Caucasian and 53 Afr. Am. (n=580) (23)	Two siblings with asthma	~10 cM ~10.4 cM	X		X			
Wjst [17;24]	German	97 (n=415)	Two siblings with asthma	~10.7 cM	X		X	X		X
Ober [20]	US – Hutterites	1 extended pedigree (n=693)	Population of 9 Hutterite colonies. All available individuals > 5 years of age	~9.1 cM (genome screen)	X		X			
Yokouchi [26]	Japanese	47 (n=197)	Two siblings with mite sensitive asthma	376 markers (~8.2 cM)	X		X			
Dizier [21]	French	107 (n=493)	A proband with asthma or two siblings with asthma	13 cM	X		X		X	X
Lee [25]	European (German, Italian, Swedish and Dutch)	199 (n=839)	Two siblings with atopic dermatitis	380 markers (~8.2 cM)		X	X	X		
Xu [7], Present study	Dutch	200 (n=1174)	A proband with asthma (symptoms, BHR to histamine between 1962 and 1975)	~10 cM			X	X		X

BHR bronchial hyperresponsiveness

evidence from other investigators demonstrating linkage to chromosome 1p, 5q, and 12q for asthma and atopy related phenotypes (table 3). Because the results on 5q[31], 6p, 7q and 12q[7] have been discussed previously, we will now discuss the relevance of linkage on 8p, 11q, 13q and 17q and relate them to positional candidate genes based on Genemap '99 and the OMIM (Online Mendelian Inheritance in Man) database.

Linkage of asthma to chromosome 8p (marker D8S1130) was observed in a conditional analysis in the US-CSGA sample, the same marker that showed linkage to a positive skin test to house dust mite in the present study. Evidence for linkage for asthma to chromosome 8p in the CSGA study increased after conditioning on the evidence for linkage to chromosome 11q21, indicating gene-gene interactions between loci at chromosome 8p and 11q in asthma.[27] We observed evidence of linkage to chromosome 8p (skin test to house dust mite) and chromosome 11q (Phadiatop and skin test to house dust mite).
Chromosome 11q13 was one of the first regions identified in linkage stu dies of atopy and asthma.[32,22] In the first 92 families in this population, we have previously resported that we did not find evidence for linkage to the number of positive skin tests, total serum IgE levels or bronchial hyperresponsiveness. This may seem contradictory, however, a positive skin test to house dust mite and Phadiatop were not analyzed in that study.[33] The region on 11q (11q21-q24) that was linked to atopy in this study, is approximately 35-45 cM telomeric to the FCERIB region at chromosome 11q13. Linkage to 11q21 was also observed in the CSGA population for the asthma phenotype[27], and to specific IgE to house dust mite in German families. [34] Candidate genes in this region include matrix metalloproteinase genes 1, 3, and 8, and the δ, ϵ, and γ subunits of CD3. CD3 is found on T-cell surfaces associated with T cell receptor α and β, that bind antigen in association with the major histocompatibility complex proteins on host cell surfaces.

Evidence for chromosome 13q was observed in the Australian and French genome screen for atopy and eosinophils, respectively (table 3) and with total serum IgE and skin test positivity in the current study. It includes the candidate gene endothelin receptor B.[21] A broad region on chromosome 17q has been implicated in three genome-wide searches, in particular for phenotypes related to the specific immune response. Skin test positivity and specific IgE as detected by the Phadiatop assay showed evidence for linkage in our study. Several candidate genes have been proposed.[21] Examples are the transcription factor STAT5A (signal transducer and activator of transcription 5A), chemokine receptor 7 and members of the family small inducible cytokines subfamily A, such as RANTES (regulated upon activation, normally T-expressed, and presumably secreted) and eotaxin. RANTES is a chemokine responsible for recruitment of inflammatory cells such as eosinophils and T lymphocytes. Promoter variants of RANTES have been associated with atopic dermatitis and skin prick tests, but not total IgE levels.[35,36] Eotaxin is a potent chemoattractant for eosinophils in inflamed tissue.

We did not observe evidence for linkage of atopic traits to some chromosomal regions that show evidence for linkage in other genome-wide screens, such as chromosome 4[22,26], 9[20,24], 11p[17,21,26,37],16 [20,22],19 [20,21], and 20[20,22]. This could reflect population heterogeneity or may be explained by a lack of power to identify all regions in every population. In atopy, different patterns of gene-gene and gene-environmental interaction may be important in different populations and for different allergic phenotypes. [27]

In conclusion, this study shows familial aggregation of atopic phenotypes. High total serum IgE levels and the presence of specific IgE to common aeroallergens do not show complete overlap in family members from probands with asthma. In addition, distinct regions on the genome are implicated for the different atopic phenotypes. Evidence was found for the presence of atopy susceptibility genes on chromosome 2q, 5q, 6p, 7q, 8p, 11q, 12q, 13q and 17q. Most of these regions have also shown evidence for linkage to atopy associated phenotypes in other genome-wide studies. Further replication and fine mapping studies will lead to identification of atopy susceptibility genes and ultimately provide information on the pathogenesis of atopic disease. Based on the clustering of linkage signals in genome wide screens on atopic phenotypes, we propose that in addition to shared genes, different genes may regulate total and specific serum IgE.

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Electronic Database information

Asthma Gene Database: http://cooke.gsf.de/asthmagen [13]
Genemap 99: www.ncbi.nlm.nih.gov/genemap99
LDB (Genome Location Database), http://cedar.genetics.soton.ac.uk
LINKAGE and CRIMAP software, http://linkage.rockefeller.edu/soft/linkage
Marshfield Center for Medical Genetics:
http://research.marshfieldclinic.org/genetics
OMIM (Online Mendelian Inheritance in Man),
www.ncbi.nlm.nih.gov/omim
S.A.G.E. (Statistical Analysis for Genetic Epidemiology),
http://darwin.cwru.edu/pub/sage.html
SOLAR (Sequential Oligogenic Linkage Analysis Routines),
http://www.sfbr.org/sfbr/public/software/solar/index.html

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Chapter 7 | Fine-mapping of an increased Total IgE susceptibility gene on chromosome 2q: Analysis of CTLA-4 and CD28

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Abstract

Asthma and allergy are complex diseases caused by an interaction between host susceptibility factors and environmental exposures. Genetic studies have identified several chromosomal regions that may contain genes that contribute to asthma, atopy, or both. Evidence for linkage of high total serum IgE levels and other allergy-associated phenotypes has been observed to a region on chromosome 2q in multiple populations. Two candidate genes in this region are CTLA-4 and CD28, which function together to regulate a key control point for IgE synthesis and regulation. T-cells recognize antigen-presenting cells by the antigen bound to MHC class II molecules, but this binding alone is insufficient to activate T-cells. Co-stimulation by other receptor-ligand complexes facilitates efficient and appropriate activation of T-cells. Two of the main co-stimulation complexes are the B7-1 (CD80) and B7-2 (CD86) ligands and CD28 and CTLA-4 receptors. We have sequenced the coding region of both of these genes and identified two novel SNPs in CTLA-4 and three in CD28. These polymorphisms, in addition to the two existing SNPs in CTLA-4, were analyzed in a well-characterized population of Dutch families ascertained through a proband with asthma that was initially studied 25 to 35 years ago. Using the probands and their spouses from this population in a case-control study design, we observed significant associations of atopy and asthma related phenotypes with two of the CTLA-4 polymorphisms. Significant evidence for an association was observed with the –1147 C/T SNP to asthma and BHR ($p = 0.005 – 0.007$), but not to the allergy-related phenotypes. The Thr17Ala SNP (+49 A/G) in exon 1 was significantly associated with all four phenotypes examined in this population, driven by individuals that were homozygous for the Thr (A) allele. These data suggest that the co-stimulation pathway, and specifically the role of CTLA-4, is important in the development of atopy and asthma related phenotypes.

Introduction

Asthma and allergy are complex diseases caused by an interaction between host susceptibility factors and environmental exposures. Bronchial hyperresponsiveness and elevated total serum IgE levels are phenotypes that predispose individuals to the development of allergy and asthma.[1-4] Genetic studies have identified several chromosomal regions that may contain genes that contribute to asthma, atopy, or both. Evidence for linkage of high total serum IgE levels and other allergy associated phenotypes have been observed in different populations: chromosomes 2q [5-8], 5q [9-11], 11q [11-13] and 12q.[14,15] Each of these chromosomal regions contains several important candidate genes that are biologically relevant to IgE regulation.

A key control point for IgE synthesis and regulation is the necessity for co-stimulation and activation of T-cells. T-cells recognize antigen-presenting cells by the antigen bound to MHC class II molecules, but this binding alone is insufficient to activate T-cells. Co-stimulation by other receptor-ligand complexes facilitates efficient and appropriate activation of T-cells. Two of the main co-stimulation complexes are the B7-1 (CD80) and B7-2 (CD86) ligands with CD28 and cytotoxic T-lymphocyte associated gene 4 (CTLA-4) receptors. CD28 is constitutively expressed on T-cells, and acts as a positive co-stimulator of T-cell activation. CTLA-4 is only expressed on activated T-cells, and acts as a negative feedback regulator of T-cell activation. For instance, CTLA-4 deficient mice have elevated immunoglobulin levels.[16] Therefore, these two receptors maintain a homeostasis in T-cell activation

Because of its role in activation and the downstream effects of this process, alteration of the co-stimulatory pathway could result in susceptibility to immunologic diseases. A polymorphism in the promoter[17]or the first exon (referred to as the +49 A/G or T17A) have been shown to be associated with type 1 diabetes[18-21], autoimmune thyroid disease[18], celiac disease[22], Grave's disease[23], and multiple sclerosis.[24;25] These studies suggest a major involvement of CTLA-4 in the pathogenesis of different immunologic diseases. In addition, CTLA-4 maps within the candidate region on chromosome 2q33 that we have reported with total serum IgE levels in a Dutch asthma cohort.[5] Association studies with a number of immunologic diseases suggest that CTLA-4 may be a susceptibility gene in the development of T-cell inflammatory responses in allergic asthma.

In a cohort of 200 Dutch families that was originally ascertained for asthma, we have performed fine-mapping on chromosome 2q with microsatellite genetic markers and analyzed CTLA-4 and CD28 as candidate genes for asthma and allergy phenotypes. In addition to previously described polymorphisms, we have identified two novel SNPs in the 5' putative promoter region of CTLA4 and three novel SNPs in the 5' region (promoter and exon 1) of CD28. We have determined and evaluated the contribution of CTLA-4 and CD28 on asthma and allergic phenotypes in this population.

Material and Methods

Population

This population has been described in detail previously.[5,9,26,27] Families were ascertained through a proband with clinical asthma initially characterized between 1962-1975. Between 1990 and 1998, 200 probands with asthma (together with their spouses, children and available grandchildren) were restu died. Briefly, all individuals underwent spirometry, bronchodilator reversibility to 800 mg albuterol, and bronchial responsiveness testing to histamine using a 30 second inhalation protocol.[27,28] For atopy, adult subjects had intracutaneous skin testing with 16 common aeroallergens and total serum IgE levels were measured. In the first 92 families, total serum IgE levels were measured by solid-phase immunoassay (Pharmacia Diagnostics, Uppsala, Sweden). Duplicate measurements were made and the mean for each subject was used. If the duplicate samples differed by more than five percent the test was repeated. In the second set of 108 families, total IgE levels were measured by enzyme linked fluorescence assay (Mini Vidas, Biomerieux, Inc.). Although entire families were ascertained for linkage studies, the probands and spouses represent an appropriate cohort for this case-control association study. Probands and spouses are similar in age, removing the confounding effect of age on changes in BHR, IgE and atopy. This study was approved by the Medical Ethics Committee at the University of Groningen and the Institutional Review Board at Wake Forest University School of Me dicine. All subjects provided written informed consent.

Molecular Methods

CTLA-4 was sequenced in 32 unrelated African-American individuals with mild and severe asthma. All four exons and 1200 bp of the 5′ putative promoter region were sequenced, using primers (Table 1) designed from the available genomic sequence (GenBank accession number AF225900). Sequencing was performed using BigDye terminator chemistry (ABI) and an ABI 3700 DNA Analyzer (ABI, Inc., Foster City, CA). Potential 5′ promoter binding sites were determined using MatInspector from Genomatix (genomatix.gsf.de/cgi-bin/matinspector/matinspector.pl) with both of the –994 and –1147 alleles. CD28 was sequenced using similar techniques and three novel SNPs were detected. Since all three of these SNPs were in linkage disequilibrium in the individuals that were sequenced, only one of these (-824 A/G) was genotyped in the entire proband-spouse population.

Microsatellite genetic markers were genotyped using fluorescently labeled oligonucleotide primers. PCR products were pooled, run on a 3700 DNA Analyzer (ABI, Inc.), and scored using Genotyper software (ABI, Inc.). A modified version of Linkage Designer (dnalab www.uia.ac.be/dnalab /ld.html) was used to bin alleles and check for inheritance inconsistencies. The output from Linkage Designer was then analyzed further for any inconsistencies by running the LINKAGE software without disease information. The final check that was performed on the data was to run CRIMAP[29] to de-

termine the order and length of the chromosomal map and to detect double recombinants. SNPs were genotyped using PCR and RFLP analysis. The primers and annealing temperatures for each specific SNP are reported in Table 1.

Table 1. Primers for sequencing and genotyping CTLA4

Sequencing primers

Name	Sequence	Location
CTLA4PR1F	5'-GCTGAGGTGTGGACAATGG-3'	5'
CTLA4PR1R	5'-TCAGGTGTTCTTAAAAGCCTTAAC-3'	
CTLA4PR2F	5'-CTTGAATCATTTGGGTTGGC-3'	5'
CTLA4PR2R	5'-AAGTGGAGACTTGGAGAATTTCC-3'	
CTLA4PR3F	5'-TGGTTAAGGATGCCCAGAAG-3'	5'/exon 1
CTLA4PR3R	5'-AGGTAGGAGAAACACCTCCTCC-3'	
CTLA4E2AF	5'-AAGCTAGAAGGCAGAAGGGC-3'	exon 2
CTLA4E2AR	5'-CACCCACAATAAGCAAGGCT-3'	
CTLA4E3AF	5'-ATGTTGGGGACTAGAGCCCT-3'	exon 3
CTLA4E3AR	5'-TCCTTCCTCTTCATTTATTGCC-3'	
CTLA4E4AF	5'-ATTTTTAACCAGCTAGGGACCC-3'	exon 4
CTLA4E4AR	5'-CATTTCGGCTATAAACGTCTCA-3'	

Genotyping Primers

SNP	Primer Sequences	Annealing Temp.	Restriction Enzyme	Reference
-1147 C/T	5'-GCTGAGGTGTGGACAATGG-3' 5'-GTTAAGGCTTTTAAGAACACCTGA-3'	60°C	*Fok* I	This report
-994 A/G	(same as -1147 C/T)	60°C	*Hsp* 92II	This report
-318 C/T	5'-AAATGAATTGGACTGGATGGT-3' 5'-TTACGAGAAAGGAAGCCGTG-3'	62°C	*Mse* I	Deichmann et al. 1996
+49 A/G (A17T)	5'-AAGGCTCAGCTGAACCTGGT-3' 5'-CTGCTGAAACAAATGAAACCC-3'	65°C	*BstE* II	Marron et al. 1997
3' UTR TA rpt	5'-fam-GCCAGTGATGCTAAAGGTTG-3' 5'-AACATACGTGGCTCTATGCA-3'			

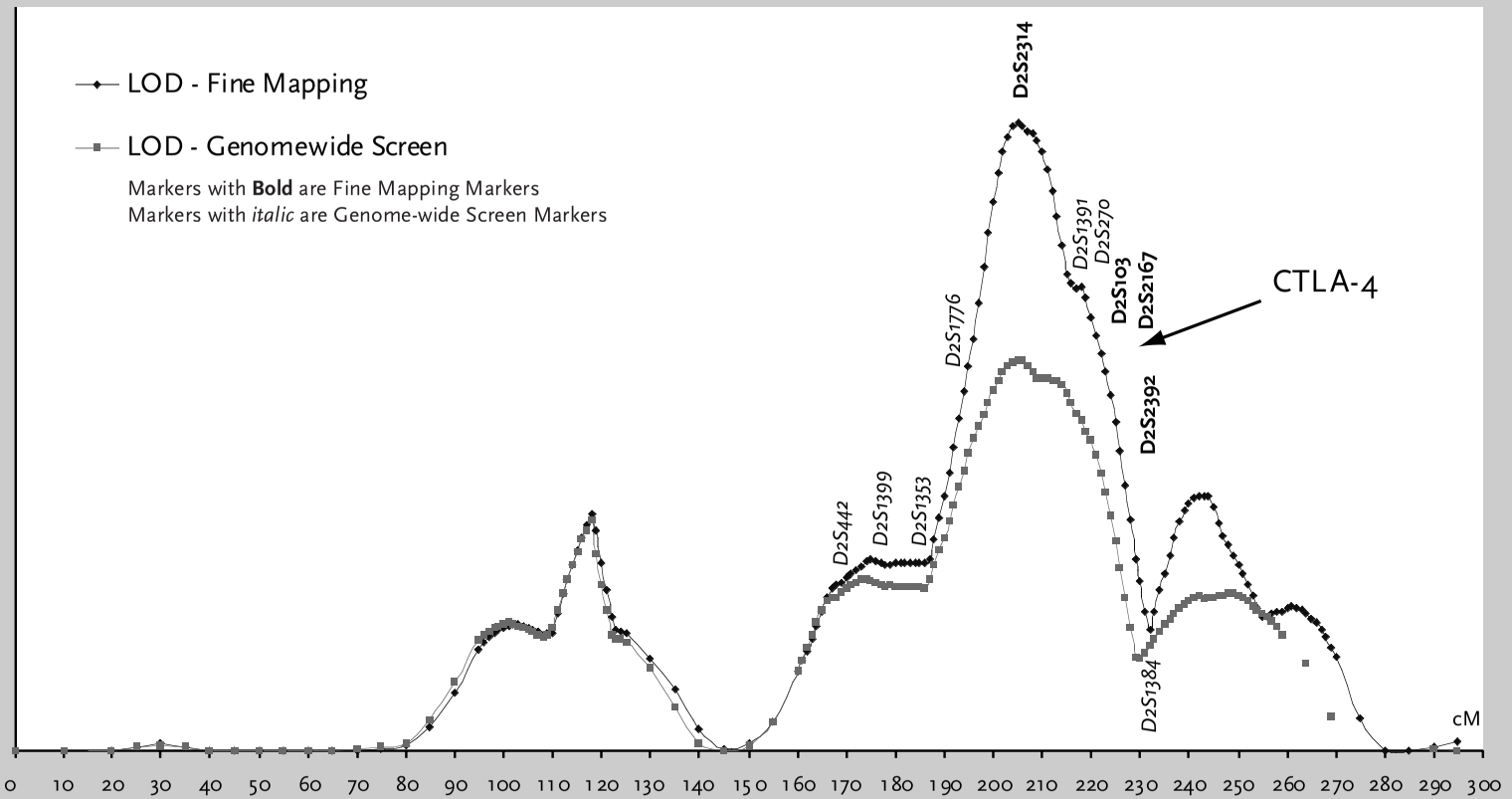
Genetic Analysis

Fine mapping on chromosome 2 was performed using an additional 9 markers and the SNPs genotyped in both CTLA-4 and CD28. Variance component linkage analysis was performed using the computer program package Sequential and Oligogenic Linkage Analysis Routines (SOLAR). The same analysis was performed for the genome wide screen for total serum IgE levels and is described in more detail in Xu et al, 2000.

Because of the previous associations of CTLA-4 with immunologic diseases, its role in co-stimulation of T-cell activation, and our linkage data suggesting an allergy susceptibility gene on chromosome 2q33 in this same population, our primary hypothesis was that polymorphisms in this gene would alter allergy phenotypes. We therefore investigated association with total serum IgE levels and skin test responsiveness to common allergens, but also examined the closely related phenotypes asthma and BHR. As described previously, all of the probands met published criteria for a diagnosis of asthma.[27] Total serum IgE was logarithm-transformed to approximate a normal distribution and analyzed as a quantitative trait. Differences between groups were tested with ANOVA, t-test, and multiple regression. Individuals were considered responsive to an allergen skin test if one or more test showed a mean wheal diameter of ≥ 5mm. For the asthma phenotype, only probands were included in the case group, whereas for BHR, cases were defined as original probands and spouses with a $PC_{20} \leq 32$ mg/ml histamine. The control group for both BHR-positive and asthma cases consisted of BHR-negative spouses ($PC_{20} > 32$ mg/ml). Each of the biallelic polymorphisms was analyzed by comparing differences in genotype frequencies between cases and controls. Chi-square tests assuming a dominant model were performed, due to the small number of homozygotes for the rare allele. No corrections were made for multiple comparisons for two reasons. First, the phenotypes tested (asthma, BHR, total serum IgE levels, and skin test response) are strongly associated with each other in this population and, therefore, the statistical analyses do not represent independent tests. Second, we performed tests for association with phenotypes that have been observed by other investigators, both to confirm previous results and to better characterize susceptibility to asthma and atopic phenotypes in our population.

Linkage disequilibrium testing between SNPs was performed using an exact test assuming multi-nominal probability of the multi-locus genotype, conditional on the single-locus genotype.[30] A Monte Carlo simulation was used to assess significance, by permuting the single-locus genotypes among individuals in the sample to simulate the null distribution. The empirical p-va lues of the LD for each pair of SNPs was based on 10,000 replicate samples.

Figure 1 Major Gene regulating age and sex adjusted Log(IgE) Levels in 200 Dutch Asthma Families.



Results

Our previous genome screen in this population revealed evidence for linkage of total serum IgE levels to several chromosomal regions, including 2q31-q33.[5] Nine additional genetic markers were added to this region and reanalyzed. The evidence for linkage on chromosome 2q33 to total serum IgE levels increased from 1.96 with the genome screen data to 3.16 with the addition of the new markers (Figure 1). With this new genetic data, the chromosome 2 linkage accounts for 36% of the total variance in total serum IgE levels in these Dutch families.

Table 2. Clinical characteristics of dutch proband/spouse population*

	Probands	Spouses
Sex, M:F	124:76	76:125
Age, mean ± SD	52.1 ± 8.4	51.0 ± 9.2
IgE		
Total IgE, IU (geometric mean)	93.0	26.2
% ≥ 100 IU/ml	72.5	15.4
Skintests		
% with ≥ 1 positive skintest	81.9	31.0
% positive with specific IgE, house dust mite	75.7	30.0
FEV_1		
% Predicted Pre-Medication (mean)	69.6	98.4
% Predicted Post-Medication (mean)	82.4	103.9
Reversibility		
%, ≥ 15% (baseline)	59.4	6.5
%, ≥ 9% (predicted)	62.9	18.9
Airway Obstruction		
% FEV_1 /VC ≤ 70% and FEV_1 ≤ 75%	51.0	3.0
BHR †		
PC_{20} ≤ 32 mg/ml, %	88.2	25.6

* Total sample population consisted of 200 probands and 201 spouses. Different numbers for the SNPs in the following tables are due to missing genotype data.
†Thirty probands were not retested due to an FEV_1 that was too low to be tested safely (FEV_1 ≤ 40% predicted).

CTLA-4 is located between the genetic markers D2S103 and D2S2392 based on data from The SNP Consortium (snp.cshl.org), placing it on the distal shoulder of the chromosome 2 linkage peak (Figure 1). Due to the functional and positional information available for CTLA-4, we evaluated it as a candidate gene for regulating total serum IgE levels. Sequencing of 1200 bp of the putative 5′ promoter region and all four exons revealed two novel single nucleotide polymorphisms, both located in the 5′ region of CTLA-4. These SNPs are identified as –994 A/G and –1147 C/T, relative to the translation start site (+1) (Figure 2). The –994 A/G substitution polymorphism was detected in the African-American asthma population (used for sequencing only), but was not detected in the Dutch probands and spouses presented in this study. The substitution of C for T at –1147 creates consensus binding sites for serum response factor (SRF) and the human CCAAT displacement protein (CDP) (Figure 3).

Figure 2 Structure of CTLA-4 gene

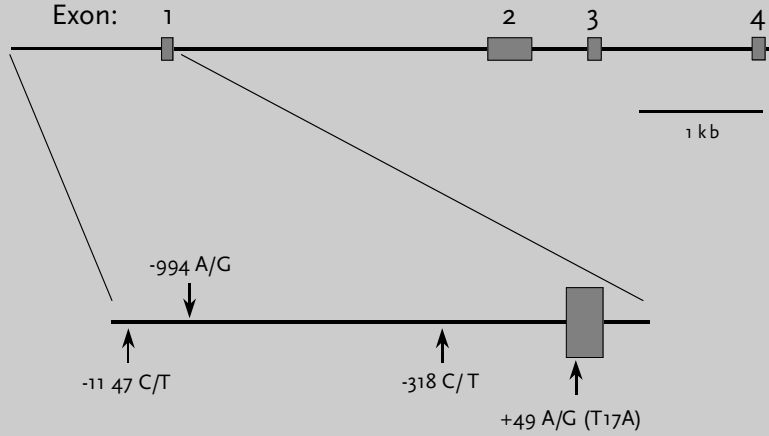


Figure 3 Substitution of -1147 A/T creating consensus binding sites

CTGAGGTGTG GACAATGGGA AACCATGGAT GGACTGGAGT AGGCAAATGT CATATTCCCT

Ik1 /2 SRF

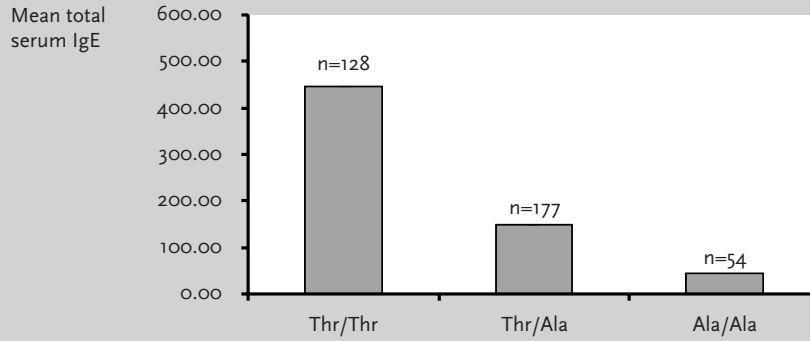
NFAT Cut

CTGAGGTGTG GACAATGGGA AACCATGGAC GGACTGGAGT AGGCAAATGT CATATTCCCT

Ik1 /2

NFAT

Figure 4 Association of the Thr17Ala snp with mean total serum IgE



The probands and spouses from the 200 Dutch families were analyzed for the three SNPs in CTLA-4. As a quantitative trait, total serum IgE levels (logarithm transformed) were significantly associated with the Thr17Ala (+49 A/G) polymorphism (p=0.0006), with Thr/Thr homozygotes having the highest total serum (log) IgE (1.84 ±0.77), Ala/Ala homozygotes the lowest (1.46 ±0.66), and Thr/Ala individuals intermediate between the two (1.65 ±0.68) (Figure 4). The overall results for the closely associated traits examined (asthma, BHR, skin test response, and total serum IgE levels) are reported in Table 3 and Figure 4. Significant evidence for an association was observed with the –1147 C/T SNP to asthma and BHR (p = 0.005 – 0.007), but not to the allergy-related phenotypes. The Thr17Ala SNP (+49 A/G) in exon 1 was significantly associated with all four phenotypes examined in this population, driven by individuals that were homozygous for the Thr (A) allele (recessive effect). Forty-six percent of individuals with total serum IgE levels of at least 100 IU/ml were Thr/Thr homozygotes, compared with 31% of individuals with total serum IgE levels less than 100 IU/ml (p=0.007). In addition, 41% of individuals with at least one positive skin test response, compared to 30% of those with none were AA homozygotes (p=0.031). A similar association was observed with asthma and BHR, where 43% of the original probands for the study were AA, compared to only 29% of their unaffected spouses (p=0.012). Similar analyses were performed for the SNP genotyped in CD28. No evidence for a significant association with allergy or asthmatic phenotypes were observed (Table 3).

Table 3. Frequency of CTLA-4 and CD28 genotypes within asthma and allergy phenotypes

	Asthma		BHR		Skin Test	
SNPs	Probands	Unaff. Spouses	$PC_{20} \leq 32$mg/ml	$PC_{20} > 32$mg/ml	≥1	0
CTLA-4						
-1147 C/T	*n=156*	*n=117*	*n=201*	*n=117*	*n=175*	*n=143*
CC	0.69	0.83	0.69	0.83	0.72	0.76
CT	0.30	0.15	0.30	0.15	0.27	0.22
TT	0.01	0.02	0.01	0.02	0.01	0.02
	p= 0.007		**p= 0.005**		p=ns	
-318 C/T	*n=176*	*n=131*	*n=176*	*n=148*	*n=195*	*n=158*
CC	0.82	0.88	0.85	0.86	0.85	0.85
CT	0.17	0.11	0.14	0.12	0.15	0.13
TT	0.01	0.02	0.01	0.01	0.00	0.02
	p=ns		p=ns		p=ns	
+49 A/G (T17A)	*n=177*	*n=134*	*n=179*	*n=151*	*n=197*	*n=162*
AA	0.43	0.29	0.41	0.30	0.41	0.30
AG	0.46	0.54	0.44	0.55	0.47	0.51
GG	0.11	0.17	0.15	0.15	0.12	0.19
	p= 0.012		**p= 0.038**		**p= 0.031**	
CD28						
-824 A/G	*n=173*	*n=129*	*n=218*	*n=129*	*n=191*	*n=156*
11	0.15	0.16	0.15	0.16	0.17	0.14
12	0.43	0.41	0.42	0.41	0.43	0.39
22	0.42	0.43	0.43	0.43	0.40	0.47
	p=ns		p=ns		p=ns	

Discussion

We have refined a region on chromosome 2q33 with evidence for linkage to total serum IgE levels. Two candidate genes for IgE regulation, CTLA-4 and CD28, are key homeostatic regulators of T-cell activation and map to this region. Two novel SNPs were identified in the 5′ putative promoter region of CTLA-4 and three novel SNPs were identified in the 5′ region of CD28. Association studies with these and two previously reported polymorphisms (-318 C/T and +49 A/G) suggest that the –1147 C/T and +49 A/G (Thr17Ala) variation in CTLA-4 are involved in the development of allergy and asthma related phenotypes in the Dutch asthma families evaluated.

Co-stimulation of T-cell activation is necessary for the downstream response of the immune response to antigen. A defective or improperly regulated co-stimulatory pathway may contribute to an allergy phenotype. Since allergy and asthma are closely related conditions, we hypothesized that the co-stimulation pathway, and specifically CTLA-4 and CD28, may play a role in the clinical characteristics of individuals in a Dutch family study that were ascertained based on a diagnosis of asthma.
Association and linkage-based studies with CTLA-4 polymorphisms have previously been performed in a variety of immunologic diseases, including asthma and atopy. In two studies, no association to either asthma or atopic asthma phenotypes was observed with polymorphisms in either CTLA-4 or CD28 in Japanese[31] or German [32]populations. In one of these studies, however, individuals were ascertained independent of their atopic phenotype[32], resulting in only 55 asthma patients compared to 205 controls. Therefore, this group may not be appropriate for evaluation of candidate genes for asthma.

In studies of other immune-related phenotypes, an association with the +49 A/G (Thr17Ala) variation has been observed. In a multi-ethnic collection of insulin-dependent diabetes mellitus (IDDM) families, the increased transmission of the G (Ala) allele to diabetic children was ethnicity dependent[20] This unequal transmission was mostly observed in Mediterranean-European (Italy, Spain and France) ($p= 10^{-5}$) and Mexican-American (p=0.002) populations. The most significant results were observed when these two groups were combined ($p=10^{-7}$).

Association of CTLA-4 with IDDM is particularly interesting because of the differences between IDDM and atopic diseases. IDDM is caused by a Th1-mediated immune response, whereas atopic diseases are mediated by a Th2 response. In addition, the majority of the reports with the +49 A/G SNP in CTLA-4 involve over-transmission or association of the G (Ala) allele with the allergy phenotype. In this study, we also observed an association with an allergy related phenotype, but with the A (Thr) allele. Association with the A (Thr) allele has also been observed in Celiac disease[33], but not with any other immune diseases. It is possible that the +49 A/G polymorphism is not the causative variant of the reported associations in these studies, but the observations are due to a nearby SNP in lin kage disequilibrium. Another possibility is that Celiac disease and elevated total serum IgE levels have a similar molecular etiology, which differs from that of autoimmune diseases. The total serum IgE levels and Celiac disease are both triggered by an environmental antigen, whereas autoimmune diseases are caused by intrinsic, organ-specific autoantibodies. Further investigation into this possibility is warranted.

Family-based linkage studies have identified the CTLA-4/CD28 region on 2q33 as containing an IgE-related gene. It is interesting to note that while allergy and asthma-related phenotypes (including total serum IgE levels) have been analyzed in numerous populations, evidence for linkage at 2q33 occurs consistently in studies utilizing European and Hispanic populations. We have recently reported evidence for linkage of total serum IgE to 2q33 in our Dutch population[5], and this region was observed with the "asthma" phenotype in our Hispanic population of the Collaborative Study on the Genetics of Asthma.[34,35] In the Hutterites, a founder population in the United States that originated in the Tyrolean Alps in the 1500s, evidence for linkage to 2q (peak marker D2S2944) was observed with positive skin test response to cockroach and house dust mite.[7] An (AT) repeat polymorphism in the 3' UTR of CTLA-4 was evaluated as a candidate gene for this region, but no association was observed to any phenotype. Analysis of the +49 A/G SNP in this population may further identify the potential role of CTLA-4. Evidence for linkage was also observed to this region of chromosome 2q with total serum IgE levels (p=0.0016) in a population of mostly German families (83 German, 5 Swedish, and 9 other nationality families).[6] A study from the French Epidemiological Study on the Genetics and Environment of Asthma, Bronchial Hyperresponsiveness, and Atopy (EGEA) also reported evidence for linkage of total serum IgE levels to 2q33.[8] Using a two-stage analysis with two sets of families, this region was significant in the first set of 46 families but was not replicated in the second set of 61.

The –1147 substitution of T for C creates a consensus binding site for the serum response factor (SRF) and the CCAAT displacement protein/Cut-like protein (CDP) (Figure 3). SRF is one component of a complex that leads to transactivation of the promoter[36]; an additional protein in this complex is one or more Ets family member. Interestingly, two genes recently described for asthma susceptibility in a Tristan da Cunha and Toronto cohort of asthma families were also Ets-related family members, referred to as ASTH1I and ASTH1J (US patent # 6,087,485). These data suggest that the SRF complex may be involved in the regulation of asthma or allergy related genes. A second potential binding site that is created by the C to T substitution is for CDP/cut, the human homologue of Drosophila cut[37], that has recently been shown to be a repressor of specific MHC class I genes.[38] If the potential CDP/cut binding site in the CTLA-4 5' region is an active site, then binding of CDP/cut may decrease transcription of CTLA-4. Down-regulation of CTLA-4 would lead to decreased repression of T-cell activation, since CTLA-4 is a negative regulator of this process. Therefore, T-cells in stimulated individuals would remain in the activated state for longer periods of time, potentially leading to allergy or asthma phenotypic expression. This is consistent with our data, where the T allele (which creates the CDP/cut site) is more common in individuals with BHR (Table 3). It is interesting to note that the CDP/cut gene is located on chromosome 7q22, the region with the highest lod score for total serum IgE levels.[5]It is possi-

ble that polymorphisms in this gene may also contribute to allergy or asthma phenotypes, either independently or by interacting with variations in other genes, such as CTLA-4.

In summary, we have identified a region on chromosome 2q33 that contains one or more susceptibility genes for asthma. Fine-mapping with microsatellite genetic markers in a Dutch population ascertained by a proband with asthma has localized this region to an interval containing multiple candidate genes, including CTLA-4 and CD28. By sequencing these two genes we have identified five new SNPs (two in CTLA-4 and three in CD28) and evaluated a subset of these SNPs for association with asthma and allergic phenotypes. Significant association was observed to polymorphisms in CTLA-4 that may regulate the expression levels, the function of the CTLA-4 protein, or both. Further analysis of this gene is necessary to determine its role in the susceptibility to asthma in this and other populations.

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Chapter 8 | Association of a promoter polymorphism of the CD14 gene and atopy

(Am J Respir Crit Care Med 2001; 163: 965-969)

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ABSTRACT

Atopy is generally considered to be caused by interaction of genetic and environmental factors. Recently, an association of a C to T transition in the promoter region of the CD14 gene on chromosome 5q31.1 and atopic phenotypes was reported in a population study of school children in the United States. The aim of the present study was to investigate the association of the C allele of the CD14/-159 with phenotypes of atopy and asthma in an adult Dutch population in which linkage of total serum IgE and bronchial hyperresponsiveness to chromosome 5q31-33 is present. We studied 159 probands with asthma and 158 spouses as controls. Phenotypes for asthma (e.g., bronchial hyperresponsiveness, physician's diagnosis) and for atopy (e.g., total serum IgE level, intracutaneous skin test, allergic rhinitis) were studied. In this population, homozygotes for the C allele had a higher number of positive skin tests and higher total serum IgE levels (in skin test positive individuals) and subsequently, more self reported allergic symptoms including rhinitis and hay fever, compared with subjects with CT and TT alleles. We conclude that the -159 C to T promoter polymorphism in the CD14 gene may result in expression of a more severe allergic phenotype.

INTRODUCTION

Atopic diseases, such as asthma, allergic rhinitis and eczema, are characterised by an elevated and prolonged immunoglobulin E (IgE) antibody response after exposure to ubiquitous, nonpathogenic allergens. Atopy is generally considered to be caused by the interaction of genetic and environmental factors.[1] There is concern about the worldwide rise of atopic diseases over the last decades and different hypotheses have been proposed to explain this increased prevalence.[2] Changes in environmental factors most likely play an important role, such as life style, diet, air pollution, allergen exposure and microbial environment.[3,4] Recent studies have suggested that bacterial infections in infancy may protect against the development of allergy.[5] In 1997, Holt and coworkers hypothesised that bacterial signals play a functional role in the maturation of the TH-1 type immune response, thereby suppressing the TH-2 type response, which may produce an atopic phenotype. Microbial products, such as lipopolysaccharides (LPS), can provide activation signals for TH-1 maturation.[6]

The pathway through which LPS may exert its function includes high- and low-affinity LPS receptors. An important high affinity receptor for LPS and other bacterial wall components is CD14, a 55-kd glycosylphosphatidylinositol anchored protein localized on monocytes, macrophages, and polymorphonuclear cells. Binding of LPS to CD14 is facilitated by lipopolysaccharide-binding protein. CD14 is also present as soluble CD14 (sCD14) in serum, where it acts like membrane bound CD14 in cells that do not express CD14.[7]

The gene encoding CD14 is a positional candidate gene for atopy, as it is localized on chromosome 5q31.1,[8] a region that has been linked to asthma and atopic responses.[9-14] In 1999, Baldini and coworkers reported the association of a polymorphism in the CD14 gene and atopy. In the promoter region of the CD14 gene, a C-to-T transition was identified at position -159 upstream from the major transcription site (CD14/-159). In a Caucasian subset (n=314) of a general population sample of 11-yr-old children from the United States, homozygotes with TT had higher serum levels of sCD14 than homozygotes with CC. In addition, among skin test positive children, homozygotes with the TT genotype had lower levels of serum total IgE and a lower number of positive skin prick tests, when compared to the pooled group of subjects carrying CC and CT.[15] This study did not assess the effect of CD14 on asthma and allergic rhinitis.

The purpose of the present study was to investigate, whether the C allele of the CD14/-159 was associated with elevated total serum IgE levels and number of positive skin tests in an adult Dutch population, in which linkage of total IgE levels and bronchial hyperresponsiveness to chromosome 5q has been previously reported.[9,12] In addition, we investigated whether this polymorphism was associated with allergic rhinitis and asthma phenotypes.

METHODS

Study population

Subjects were selected from a family study on the genetics of asthma in the Netherlands, which has been described in detail by Panhuysen and cowor kers.[16] Between 1962 and 1975, patients with asthma were evaluated for diagnosis of asthma and optimization of their treatment in Beatrixoord, Haren, the Netherlands. For inclusion in this study, from this first evaluation patients had to meet three criteria: (1) symptoms consistent with asthma; (2) age ≤ 45 yr; (3) bronchial hyperresponsiveness to histamine (PC_{20} ≤ 32 mg/ml using the de Vries 30 seconds inhalation method).[17]

From 1990 onwards, these probands were restudied together with their spouses, a minimum of two children and, if possible, grandchildren. In total, 200 two- and three generation families have been studied. Using a case-control approach, probands and their spouses were selected from these families. Data are presented from 159 probands and 158 spouses, from whom DNA was available at the time of this study.

Clinical assessment

The first evaluation (1962-1975) included the performance of intracutaneous skin tests with common aeroallergens, pulmonary function testing with a water-seal spirometer (Lode Spirograph, Groningen, the Netherlands), and testing for bronchial hyperresponsiveness with histamine, using the 30 seconds inhalation protocol as described by de Vries and coworkers.[17]

At the second evaluation (1990-1998), these measurements were repeated in the probands, and also performed in the relatives. Reversibility was tested by repeating spirometry 20 minutes after administration of 800 mg of salbutamol (albuterol). All participants were asked to stop pulmonary medication before the clinical testing if possible: inhaled corticosteroids were stopped for 14 d, inhaled long acting beta-mimetics and oral antihistamines 48 h, inhaled short acting beta-mimetics and anticholinergics 8 h. The asthma patients did not have an asthma exacerbation or require a course of oral prednisone in the 6 weeks prior to the study.

This evaluation further included a modified version of the British Medical Council questionnaire with additional questions on symptoms and therapy of asthma and allergy.[16] By definition, a physician's diagnosis of asthma was present in the probands. In the spouses, it was present if the subject reported (1) being under current regular treatment for asthma, (2) having ever visited a general practitioner or specialist for asthma or (3) having ever used asthma medication. Allergic rhinitis was defined as a positive answer to one of the following questions: Do you have a runny or stuffed nose when you are near (1) animals (e.g., dogs, cats, horses), feathers (e.g., in pillows), a dusty part of the house, trees, grasses, and flowers? Hay fever was defined as a positive answer to the question: Did you ever have hay fever?

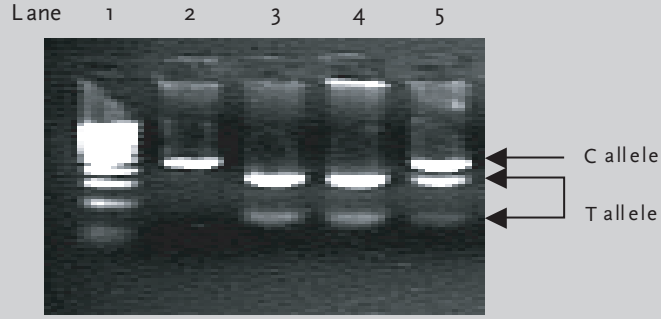
Serum total IgE was measured in the first 92 families by solid phase immunoassay.[16] In the second set of 108 families, serum IgE levels were mea-

sured by an enzyme linked fluorescent assay (Mini Vidas, Biomerieux Vitek Inc., Marcy, France). Skin testing was performed by an intracutaneous skin test with 16 common aeroallergens, a positive control, and a negative control. The following allergens were tested: mixed grass pollens, two mixed tree pollens, mixed weeds, house dust mite, storage mite, cat-, dog-, horse-, rabbit/guinea pig dander, feather mix, and five moulds *(Aspergillus Fumigatus, Alternaria Alternata, Cladosporium Herbarum, Penicillum Notatum, Botrytis Cineria)*. (ALK-Abello, Nieuwegein, the Netherlands). A positive skin test was considered to be present if the largest wheal diameter was ≥ 5 mm.
The Medical Ethics Committee of the University Hospital of Groningen and the University of Maryland approved this study. Written informed consents were obtained from all participants.

Molecular methods

DNA was extracted using standard protocols. (Puregene kit; Gentra Systems Inc., Minneapolis, MN). Genotyping of the CD14/-159 polymorphism was performed according to the protocol described by Baldini and coworkers.[15] Briefly, polymerase chain reaction (PCR) was performed in 10 ml volumes consisting of 60 ng of DNA, 250 mM dNTP, 1.5 mM MgCl, 10X buffer (Life Technologies, Rockville, MD), 0.5 U of Taq polymerase and 0.1 mM of primer 5'GCCTCTGACAGTTTATGTAATC3' and primer 5'GTGCCAACAGATGAGGTTCAC3'. Cycling conditions were 94˚C for 3 minutes, 28 cycles of 94˚C for 30 seconds, 57˚C for 30 seconds, 72˚C for 30 seconds and a final extension of 72˚C for 6 minutes. PCR amplified DNA was digested with 5 U of Ava II and 1 mL of the manufacturers buffer (New England Biolabs Inc., Beverly, MA) at 37˚C for two h. Products were fractioned on 2.0% agarose gel. Ava II digests the PCR product only when the T allele is present. The uncut product is 497 bp while the diges ted products are 144 and 353 bp (figure 1) The results of this restriction fragment length polymorphism assay were confirmed by direct sequencing of the -159 promoter region of the CD14 gene in 32 patients and controls.

Figure 1



CD14/-159 restriction fragment assay. Electrophoresis gel showing size markers on lane 1. On lane 2, one band is visible for homozygotes carrying two C alleles. On lane three and four two bands for homozygotes carrying two T alleles, and on lane five a CT heterozygote is shown

Statistical methods

Three different genetic models were tested. To study a recessive model for the C allele, CC homozygotes were compared with CT heterozygotes and TT homozygotes. To study a recessive model for the T allele, TT homozygotes were compared with individuals with CT and CC. To study a codominant model, the three genotype groups were analyzed separately.

Both parametric (T-test, ANOVA) and non-parametric analyses (Mann-Whitney, Kruskal Wallis test) were used to study phenotypic differences in each genotype group, depending on the normality of the distribution of the variables. The number of skin tests was not normally distributed, whereas serum IgE levels were log transformed to obtain a normal distribution. Linear and logistic regression analyses were used to test and correct for known confounding variables, such as age, sex and smoking. Results were considered significant if $p<0.05$ (two-sided). All calculations were performed with the SPSS 8.0 statistical package.

Table 1. Baseline characteristics of the study population at the second evaluation

Characteristic	probands (n=159)	Spouses (n=158)
Male, %	63.5	36.7
Age, years (mean, range)	52.4 (37-76)	51.3 (34-76)
Smoking (none, ex-, current)	55/63/41	42/54/62
Asthma (doctor's diagnosis), %*	100	8.7
Allergic rhinitis (self reported), %*	59.1	19.6
Hay fever (self reported), %*	31.4	5.7
FEV_1% predicted (mean, SD)	67.7 (25.2)	97.8 (13.1)
Bronchial hyperresponsiveness (PC20 ≤ 32mg/ml), %	90.2	25.6
Skin test positivity (minimum 1 positive), %	79.9	29.1
Number of positive skin tests (median, range)	3.5 (0-13)	0 (0-9)
Serum total IgE, IU/ml (geometric mean, 95% CI)	97.3 (76.0-124.5)	27.4 (21.6-34.6)

Skin test was not available in 1 proband. IgE was not available in one other proband, FEV_1 not available in 3 probands. Testing of bronchial hyperresponsiveness was not performed in 29 individuals (27 probands, 2 spouses) due to low baseline lung function. All probands were hyperresponsive at the time of initial testing. PC20 ≤ 32 mg/ml with 30 seconds inhalation protocol. * For definition see methods

RESULTS

Study population

Baseline characteristics of the study population at the second evaluation (1990 - 1998) are shown in table 1. Probands were predominantly male and their mean age was 52 years. All probands were hyperresponsive at initial testing. Although probands were not selected for atopy, 79.9% had atopy as measured by a positive skin test, compared to 29.1% of the spouses.

Genotype frequency of the CD14/-159 polymorphism

The genotype frequencies in probands were 32.1% for CC homozygotes, 47.8% for CT heterozygotes and 20.1% for TT homozygotes. In the spouses, these frequencies were 19.6%, 53.8%, and 26.6%, respectively.

Association of the C allele with total IgE levels, skin tests, hay fever and allergic rhinitis

Based on the report of Baldini et al.[15], the primary analysis was the association of CD14/-159 and skin tests and serum total IgE levels. In the skin test positive population, CC homozygotes had significantly higher serum IgE levels compared to CT heterozygotes and TT homozygotes (p=0.036) The difference between the latter was not significant in skin test negative individuals (table 2).

Table 2. Association of the CD14/-159 promoter polymorphism and total serum IgE levels

	Skin test negative		Skin test positive	
Genotype	n	Total IgE levels	n	Total IgE levels
CC	31	20.3 (11.3-36.5)	51	162.6 (104.7-252.6) *
CT	71	20.8 (15.1-28.8)	88	94.5 (69.9-127.7)
TT	41	17.5 (12.0-25.4)	33	105.9 (66.8-167.7)

Total IgE levels are expressed as geometric mean (95% confidence interval of the mean). * $p < 0.05$ for CC versus CT and TT

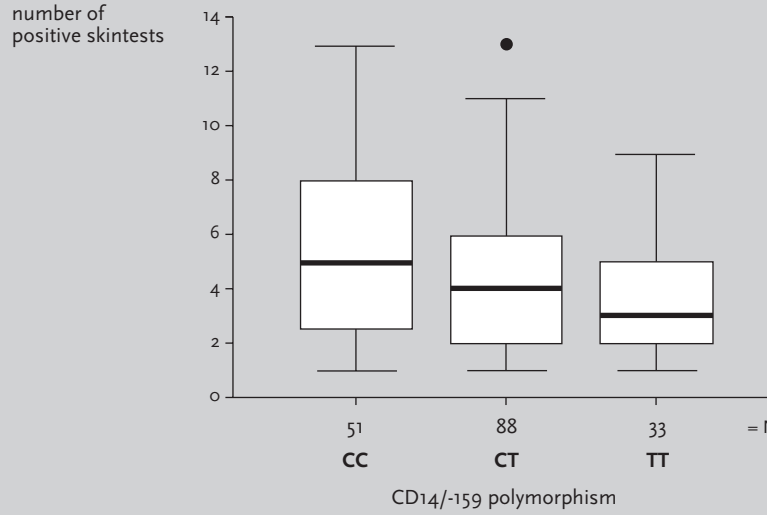
In the skin test positive population as well as in the total population, CC homozygotes had a higher number of positive skin tests compared to CT heterozygotes and TT homozygotes. In skin test positive individuals, the median (mean) number of positive skin tests in individuals with CC was 5 (5.61), for CT 4 (4.19) and for TT 3 (3.76) (figure 2). The difference was statistically significant in a codominant model (p =0.01). This difference was also significant in a recessive model for the C allele (p=0.008) (figure 2).
In a logistic regression analysis, CC homozygotes had a higher frequency of reporting hayfever (odds ratio (OR) 2.15, 95% confidence interval (CI) 1.18-3.92 and allergic rhinitis (OR 1.80, 95% CI 1.08-2.99), compared to individuals carrying CT and TT. After introduction of skin test positivity in three classes (0, 1-4, ≥5 positive skin tests) into this regression analysis, this association was no longer significant (allergic rhinitis: CD14 CC homozygotes OR 1.54, 95% CI 0.89-2.67; skin test positivity OR 2.53, 95% CI 1.88-3.43; hay fever: CD14 CC homozygotes: OR 1.78, 95% CI 0.92-3.45; skin test positivity OR 3.61, 95% CI 2.39-5.44).

Association of the C allele with asthma

The CD14/-159 genotype was not associated with doctor's diagnosed asthma and bronchial hyperresponsiveness to histamine, and peripheral blood eosinophilia ($p > 0.05$). In the total population of asthmatics and their spouses, the CD14 genotype showed borderline significance with prebron-

chodilator FEV_1 expressed as percentage of predicted in a codominant model (p=0.05) and in a recessive model for the C allele (p=0.015). The CD14/-159 was not associated with FEV_1 level when analyzed within the group of asthma patients. Reversibility to a bronchodilator expressed as the change in FEV_1 was not related to the CD14 genotype neither in the total nor in the asthmatic population.

Figure 2 Boxplot of number of positive skintests within skintest positive individuals.



This boxplot shows medians and interquartile ranges. P<0.01 for CC versus CT and TT (recessive model) ; p<0.05 for CC versus CT versus TT (codominant model).

DISCUSSION

This study confirms an association of the CD14/-159 promoter polymorphism with the number of positive skin tests and total serum IgE levels in skin test positive individuals. In this population selected for probands with asthma, as well as in the population studied by Baldini et al., the CD14/-159 genotype was not associated with atopy defined by at least one positive skin test.[15] Therefore, the CD14/-159 genotype does not appear to represent a susceptibility gene for the development of atopy, yet it appears to produce a more severe atopic phenotype, that is, a higher total number of positive skin tests and a higher total IgE level in skin test positive individuals.

In the population of children as described by Baldini et al., the C allele of the CD14/-159 polymorphism had a dominant effect on total IgE levels and the number of positive skin tests.[15] In our data, the C allele is associated with a higher number of positive skin tests in a codominant model, and with higher total IgE levels and higher number of positive skin tests in a recessive model. The results confirm the importance of the C allele

influencing the expression and severity of the atopic phenotype. One might argue that this difference in the genetic model suggests that these data do not confirm the previous association study completely. However, we regard this finding as a confirmation, given the same direction of the phenotypical effects of the C allele (higher number of skin tests, higher serum IgE levels). The unresolved difference between these studies is the influence of having one C allele since the difference between the genetic models is the grouping of the heterozygotes. Possible explanations include differences in recruitment strategies of these populations, an age effect (mean age of our population is 52 years versus 14 years in the study of Baldini), or different gene-gene, and gene-environmental interactions in these different populations.

We could not find a significant association of CD14/-159 with phenotypes of asthma, such as bronchial hyperresponsiveness and physician's diagnosis of asthma. These data fit our hypothesis that separate genes appear to regulate susceptibility to high total serum IgE levels and bronchial hyperresponsiveness on chromosome 5q.[12] Atopy and asthma, while seperate disease entities, are closely related and it is difficult to distinguish their specific expression on the clinical phenotype. Several studies have shown that total serum IgE levels as well as the number of positive skin prick tests are associated with the presence of bronchial hyperresponsiveness, the development of asthma symptoms and a physician's diagnosis of asthma.[18,19] Due to this close interrelation of asthma and atopy, alleles that are associated with atopy will be overrepresented in asthmatic individuals when compared with the general population. It is possible that genetic studies that seperate the effects of atopic and asthmatic genotypes may increase our basic understanding of how allergic factors affect the development and progression of asthma.

A genetic association may be confounded by population admixture or can be observed as the result of linkage disequilibrium with another allele.[20] Since all individuals in our study are white and from a relatively homogeneous population in the northern part of the Netherlands, association due to population admixture seems very unlikely. Linkage disequilibrium as a possible confounder cannot be excluded. However, other polymorphisms in the promoter region of the CD14 gene have not been identified to date.[15,21] The same promoter polymorphism was reported by Hubacek and coworkers in a study on myocardial infarction (-260 C to T transition). CD14/-159 is 260 base pairs upstream from the major translation region of CD14 (J. A. Hubacek, personal communication). Interestingly, in this study the T allele had a higher frequency in survivors of myocardial infarction compared to a control group indicating a possible role of CD14 in atherosclerosis.[22]

Hall[23] suggested four criteria for a specific gene to become an established biologic candidate gene in a complex disease: (1) consistent association; (2) location of the gene in a chromosomal area of linkage; (3) change in protein level or function by the mutation and, finally, (4) biological plausibility of the gene for the disease.

Two previous papers have reported association of CD14/-159 with serum total IgE levels.[15,24] Apart from the paper by Baldini and coworkers, Gao and

coworkers[24] studied the association of this polymorphism in a British (n=300) and Japanese population (n=200). The CD14 genotype appeared to be associated with total IgE levels in RAST-negative individuals in a recessive model for the C allele in the British sample. This finding differed from the results in skin test-positive individuals in the US and the Netherlands. These authors concluded that CD14 might not be an atopy locus, but may act as a basic modifier of serum total IgE levels.

The CD14 gene is localized on chromosome 5q31.1, an area consistently reported to be linked to phenotypes of asthma and atopy. However, linkage analysis for a modifier effect within skin test positive family members has not been performed.[9,12]

The functional role of CD14/-159 was indicated by the finding that CC homozygotes have lower levels of sCD14 and a lower density of CD14 on monocytes than TT homozygotes.[15,22] This suggests that CD14/-159 could influence the transcription rate of the CD14 gene. CD14/-159 is near an SP1 binding site that has a major influence on monocyte specific expression of CD14, and near a CCAAT/enhancer-binding protein site that may play an important role in promoter activation of the CD14 gene during monocyte development.[25,26] In addition, CD14/-159 is one basepair upstream from a putative AP-2 binding site.[25] More functional studies are needed to study the effect of CD14/-159 on promoter activity.

Finally, is CD14 a plausible biologic gene for atopy? CD14 is a multifunctional receptor and may play a role in different biological and pathophysiological processes: apoptosis, sepsis, and inflammatory diseases, such as atherosclerosis and atopy. [5-7,27,28] There are several plausible explanations for a possible role of CD14 in atopy. CD14 on monocytes and polymorphonuclear cells functions as a receptor for LPS, thereby inducing mediator and cytokine release, including IL-6 and IL-8. Blockade of CD14 prevents release of LPS induced cytokines. Thus, CD14 may be involved in a pro-inflammatory pathway through the release of cytokines.[7] In addition, Jabara and Vercelli[29] suggested a direct action of CD14 on monocytes, thereby inhibiting IgE production by B-lymphocytes. Finally, Holt and coworkers[6] suggested that LPS or other bacterial wall products could stimulate antigen presenting cells (APCs) (e.g., dendritic cells) to produce IL-12 through the action of sCD14.[30] The source of LPS could be either the microbial flora from the gastro-intestinal tract, inhaled LPS from house dust, or bacterial infection.[6] Subsequently, genetically determined higher levels of sCD14 in serum[15], or a higher density of membrane bound CD14 could result in a stronger IL-12 signal of these APCs, which in turn may be a potent signal for Th1 maturation in early life. The interesting question is whether CD14-LPS interactions have a relevant role in the development of atopy. If this is true, the interaction of age, infectious exposure as well as environmental factors such as allergens needs to be investigated.

Besides the possible role of CD14 in the development of atopy, we studied CD14 genotype in relationship to asthma severity. Several studies have indicated that LPS inhalation in patients with asthma is associated with the seve rity of asthma, as assessed by medication use, clinical scores and an increased

bronchial responsiveness to histamine.[31-33] From studies of broncheoalveolar lavage, it has been suggested that CD14 mediated cell activation could play a role in the inflammatory response after allergen challenge.[34,35] We asked if genetic variation in the CD14 gene could therefore play a modifying role in asthma severity. In the combined sample of cases and controls, evidence for an association of CD14/-159 with prebronchodilator FEV_1 levels was identified. This association was not statistically significant in the group of asthma patients only. One reason for this may be lack of power. We conclude that these analyses need to be repeated in larger sample size before definitive conclusions can be drawn.

In summary, in this study the association of a -159 C to T promoter polymorphism and atopy is confirmed. Homozygotes for the C allele had higher number of positive skin tests and higher total serum IgE levels (in skin test positive individuals) and subsequently, more self reported allergic rhinitis and hay fever.

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Chapter 9 | Identification and association of polymorphisms in the Interleukin 13 gene with asthma and atopy in a Dutch population

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ABSTRACT

Asthma and atopy are related conditions that may share similar genetic susceptibility. Linkage studies have identified a region on chromosome 5q that contains biologic candidates for both asthma and atopy phenotypes, inclu ding several proinflammatory cytokines. Interleukin 13 (IL13), one of the candidate genes in the region, is directly involved in the regulation of IgE and has been associated with both asthma and atopy. We sought to identify new polymorphisms in the IL13 gene, and evaluated the involvement of a subset of these variants in asthma and atopy in a case-control study using probands and spouses from a Dutch asthma family study. IL13 was sequenced in 20 probands and 20 unaffected spouses, and ten polymorphisms were identified, 4 novel and 6 previously reported. Three SNPs were detected in the 5'-promoter region, 2 in intron 1, and 5 in exon 4. Only one of the exon 4 SNPs resulted in an amino acid change (Arg130Gln). We analyzed three single nucleotide polymorphisms (SNPs) in IL13 in an extended group of 184 probands and their spouses: one in the promoter region (-1111), the Arg130Gln (nucleotide position 4257), and a 3' UTR SNP (nucleotide position 4738). The most significant associations were observed to asthma (p=0.005), bronchial hyperresponsiveness (p=0.003), and skin-test responsiveness (p=0.03) with the –1111 promoter. These results provide evidence that variation in the IL13 gene is involved in the pathogenesis of asthma and atopy. Further investigation is required to determine which specific alleles or combination of alleles contribute to these phenotypes, and the possible downstream effects of the resulting change in IL13 levels or activity.

INTRODUCTION

Asthma is a common respiratory disease characterized by intermittent airways obstruction and respiratory symptoms that are caused by acute and chronic bronchial inflammation. Bronchial hyperresponsiveness (BHR) and total serum IgE levels are closely associated with the asthma phenotype and have a strong genetic component.[1-6] It has been well documented that the presence of atopy and BHR may precede the development of clinical asthma.[1-2] The development of asthma appears to be determined by the interaction between host susceptibility (genetics) and a variety of environmental exposures.

Numerous genetic studies have mapped an asthma and/or atopy susceptibi lity gene(s) to a region on chromosome 5q31-q33 in several populations (Dutch[5,7], Amish [8], American Caucasian[9], Hutterite[10], and British[11,12]) This region contains a cluster of proinflammatory cytokines important in immune regulation. Two members of this cluster, interleukin (IL) 4 and IL13, have been both genetically and functionally implicated in the pathogenesis of asthma and atopy.[13-17] These cytokines are produced by Th2 cells and are capable of inducing isotype class-switching of B-cells to produce IgE.[18] They also share a receptor component, IL4Rα, which has been shown to be an important factor in the development or expression of atopy and asthma.[19-22] Furthermore, both IL4 and IL13 mRNA and protein have been localized to the airways in allergic asthma.[23,24]

The IL13 receptor consists of one IL4Rα subunit and either a low-affinity IL13Rα1[25] or a high-affinity IL13Rα2 subunit.[26] The complete receptor for IL4 is composed of one IL4Rα subunit and an IL4Rγ subunit. Therefore, it is possible that different polymorphisms in these receptors, as well as in the IL4 and IL13 cytokines, contribute to the complex regulation of atopy or asthma phenotypes. Association studies with polymorphisms in IL13 have been performed using various atopy and asthma phenotypes in several populations. A promoter polymorphism was identified at position –1111 (referred to as position –1055 in their report) adjacent to the nuclear factor of activated T cells (NFAT) site and reported to be associated with allergic asthma in a Dutch population.[14] In addition, an Arg130Gln polymorphism in exon 4 has been shown to be associated with high total serum IgE levels [15,16], atopic dermatitis[15], and asthma[17] in German[15,16], American[16], British[17] and Japanese [17] populations. In an effort to further understand the contribution of IL13 to asthma and atopy phenotypes, we have sequenced the IL13 gene in probands with asthma and their unaffected spouses to identify new sequence variants. We also performed case-control association studies with three of these polymorphisms in this Dutch population, in which we have previously obtained evidence for linkage on chromosome 5q31 to bronchial responsiveness and total serum IgE levels.[5,6]

MATERIALS AND METHODS

Population

This genetically homogeneous population has been described in detail previously[5,7,27] Probands (local Caucasian Dutch patients with asthma) were originally studied between 1962-1975 at Beatrixoord Hospital, Haren, the Netherlands. At that time patients were diagnosed with asthma by the presence of characteristic symptoms, airways hyperresponsiveness, and reversibility of airway obstruction. Between 1990 and 1999, probands with asthma were restudied, together with their spouses (all Caucasians), children and available grandchildren. Briefly, all individuals underwent baseline spirometry and reversibility to 800 mg albuterol; bronchial responsiveness testing to histamine was performed using a 30 second inhalation protocol.[27,28] A subject was considered to display BHR if the provocative concentration of histamine producing a 20% fall in FEV_1 (PC_{20}) was < 32 mg/ml histamine. For atopy, subjects had intracutaneous skin testing with 16 common aeroallergens, which was considered positive if the maximum wheal diameter was > 5mm. In the first 92 families, total serum IgE levels were measured by solid-phase immunoassay (Pharmacia Diagnostics, Uppsala, Sweden). Duplicate measurements were made and the mean for each subject was used. If the duplicate samples differed by more than five percent the test was repeated. In the second set of 108 families, total IgE levels were measured by enzyme linked fluorescence assay (Mini Vidas, Biomerieux, Inc.). Although the entire families were ascertained for genetic linkage studies, the probands and spouses represent an appropriate cohort for case-control association studies, especially since they are of comparable ages and have experienced similar environmental exposures, which accounts for some of the known age related differences in the frequency of BHR, serum IgE levels, and skin-test responsiveness. A total of 184 probands and their spouses were used for this IL13 association study. This study was approved by the Medical Ethics Committee at the University of Groningen. All subjects provided written informed consent. DNA was isolated from lymphocytes using standard procedures.

DNA resequencing of IL13

Resequencing of the IL13 gene in 20 affected (probands) and 20 unaffected individuals (spouses) was performed by cycle sequencing of overlapping PCR-amplified DNA fragments covering the 5'-flanking region, exons 1 to 4 and intron 1. We decided on this sequencing strategy to identify SNPs that are most likely to alter the regulation or functional activity of IL13. Primers and fragment sizes are shown in Table 1 (PCR primer pairs were designed using Primer Express™ version 1.0; Perkin-Elmer Applied Biosystems). PCR amplifications were carried out in 10 ml volumes containing 1X GeneAmp® PCR buffer (Perkin Elmer Applied Biosystems), 20 ng of genomic DNA, 30 ng of each forward and reverse primer, 400 mM of each dNTP (Amersham-Pharmacia Biotech), 1.5 to 3 mM $MgCl_2$ (primer-depen-

dent) and 0.5 units AmpliTaq Gold™ (Perkin Elmer Applied Biosystems). Before sequencing, amplification products were incubated with shrimp alkaline phosphatase (0.5 units; Amersham Pharmacia Biotech) and exonuclease I (5 units; Amersham Pharmacia Biotech) at 37°C for 30 min, followed by heat inactivation at 80°C for 15 min. Amplification products were double-strand sequenced using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems). After cycle sequencing, unincorporated BigDye terminators were removed using Sephadex G-50 (Sigma) in Millipore multiscreen HV plates. Sequence reactions were analyzed using an ABI PRISM 377 sequencer (Perkin Elmer Applied Biosystems) and the resultant chromatograms were aligned and viewed using Phred/Phrap [29] and Consed [30] software. The GenBank accession numbers for the reference genomic and mRNA sequences used for the IL13 gene are reported in the footnote to Table 1.

Table 1. PCR Primers Used in Resequencing the IL13 Gene*.

Alias	Forward Primer (5'-3')	Primer Pos. *	Reverse primer (5'-3')	Primer Pos.*
IL13 Prom.1 †	CCTTGGTCCCTGGAGACCAC	85 - 104	CCATGTCGCCTTTTCCTGCT	1112 - 1131
IL13 Prom.2	GTTGAGCCCCATCGAGGAC	933 - 951	GGGTCTCACTATGTTGCCTGC	1448 - 1468
IL13 Prom.3	CCTAGGCAGGCAACATAGTG	1443 - 1462	GCTATGGGAATTTGGGGAGT	1722 - 1741
IL13 Prom.4	TTTAAGAGACTGGTTCATCG	1692 - 1711	ACTTATTGAGAAGGGTCCAG	1972 - 1991
IL13 Prom.5	TAAACCCACCCAGATCTTGG	1942 - 1961	TGGTCAACAAAAGCGCCATG	2213 - 2232
IL13EX1	GCTGCCACAAGACGCCAAGGCC	2134 - 2155	CCCTCATAGCTAGGACCCTGGC	2360 - 2381
IL13IN1.1	CCCTGTGCCTCCCTCTACAGCCC	2276 - 2298	GGAGAGCAGGTAGTCCCTGGGG	2558 - 2579
IL13IN1.2	CTGGGCTGGGGGGCTCAGC	2504 - 2522	CTCTACTAACGAATCCTCCTG	2744 - 2764
IL13IN1.3	GCAGCCATTCTCCCCAGCACC	2705 - 2725	CATGGACACTCTGGTGTTGGC	2946 - 2966
IL13IN1.4	GTGAGGTTAAGTGACAGAGG	2908 -2925	GTAAGGGACAAGCTGCATGC	3146 - 3165
IL13IN1.5	CCCGCAGGCCCCTGTCCTCCTG	3104 - 3125	GGTCTGCCCCAGCACAGGCC	3313 - 3332
IL13IN1.6	CTGCCAGGCCTGCCTCTGTG	3272 - 3291	CAGCTGTCAGGTTGATGCTC	3432 - 3451
IL13EX2	GCCAGCACTCTGCTCACTGTCAC	3367 - 3389	GCCCCATCCTCCCTGCACCC	3469 - 3488
IL13EX3	CCCAAGCAGGGCCTGACCCCTCGG	3669 - 3692	GCAGGGTGGGTGTGAGAGGG	3829 - 3848
IL13EX4.1	GGCGTTCTACTCACGTGCTGACC	4123 - 4145	GCTAAGGAATTTTACCCCTCCC	4377 - 4398
IL13EX4.2	GTCTTGGGTAGGCGGGAAGG	4350 - 4369	CCTGTGTGTGAAGTGGGTCC	4604 - 4623
IL13EX4.3	CCTTGCCAGACATGTGGTGGG	4579 - 4599	CGGATGAGGCTCCGAGGCC	4854 - 4872
IL13EX4.4	CTGCTACCTCACTGGGG	4831 - 4847	CCCCAAAGGCCAAAATGAAAGAC	5111 - 5133

* Genbank accession number U31120 was used as the reference sequence.
† To facilitate sequencing of this amplification product, the following forward and reverse nested primers pairs were used:
5'-TCTCCCGTTACATAAGGCCACC-3' (433-454) and 5'-CTGACTCCCAGAAGTCTGCCC-3' (759-779)
5'-AGCTTCGAGTGTGGACAGAGAGG-3' (800-822) and 5'-GTGGCCTTATGTAACGGGAGATG-3' (431-453)

Genotyping of IL13 polymorphisms

Three single nucleotide polymorphisms (SNPs) were genotyped in the IL13 gene using the following methods. SNP 3 (Arg130Gln) was genotyped in PCR-amplified genomic DNA by allelic discrimination using TaqMan™ technology (Perkin Elmer Applied Biosystems) on the ABI PRISM™ 7700 sequence detector (Perkin Elmer Applied Biosystems). Oligonucleotide probes homologous to the wildtype (5′-TCGCGAGGGACGGTTCAACTGAAA-3′; labelled with FAM 5′-reporter dye) and SNP (5′-TCGCGAGGGACAGTTCAACTGAAA-3′; labelled with TET 5′-reporter dye) se-

quences, and forward (5′-TAAAGGACCTGCTCTTACATTTAAAGAAA-3′) and reverse (5′-TCGAAAGCATCATTATTTGCAGAGACAGG-3′) PCR primers were designed using Primer Express™ (version 1.0; Perkin-Elmer Applied Biosystems) and synthesized by Perkin Elmer Applied Biosystems (Warrington, UK). Allelic discrimination reactions were carried out on 20 ng samples of genomic DNA in a 25 ml reaction containing 50-900 nM of each forward and reverse PCR primer, 50-200 nM of each FAM and TET probe, and 1X TaqMan® Universal PCR Master Mix (Perkin Elmer Applied Biosystems). PCR cycling conditions on the ABI PRISM™ 7700 were as follows: 50°C for 2 minutes; 95°C for 10 minutes; followed immediately by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

For SNP 6 (-1111 promoter) and SNP 7 (nt 4738, 3′-UTR), PCR was performed in a 10ml volume consisting of 60ng DNA, 0.4 mM each primer, 50mM KCl, 10mM Tris, 0.2mM each dNTP, 1.5 mM MgCl2, and 0.1 U Taq DNA polymerase. The promoter polymorphism was amplified using the primers 5′-ATGCCTTGTGAGGAGGGTCAC-3′ and 5′-CCAGTCTCGCAGGATCAACC-3′. The PCR products were then purified with Qiagen PCR purification kit and sequenced using the given primers with Big Dye kit and the 3700 DNA Analyzer (ABI). The sequence was performed in both directions and analyzed and viewed with Phred/Phrap[29], and Consed.[30] Genotypes were determined by visual inspection of the sequence files.
The 3′ UTR polymorphism was amplified using the primers 5′-CTTTGCTAACATATTTAATATTTAAATACG-3′ and 5′-GTCACCGTTGGGGATTGGGGAAG-3′. PCR cycling conditions were as follows: 94°C for 4 minutes, 30 cycles at 94°C for 30 seconds, 68°C 30 seconds and 72°C for 30 seconds with a final extension step of 72°C for 6 minutes. PCR products were digested with NheI (New England Biolabs) and the alleles resolved by electrophoresis on a 2% agarose gel. The fragment sizes were 289 bp for the G allele, and 252 bp and 37 bp for the A allele.

Genetic Analysis

Analysis was performed for 4 phenotypes including asthma and associated phenotypes: BHR, total serum IgE levels, and skin-test responsiveness. Genetic analysis was conducted with each of the biallelic polymorphisms by comparing differences of allele and genotype frequencies between cases and controls. For comparing the allele frequencies between cases and controls, chi-square tests were used. When genotypic frequencies were compared between cases and controls, chi-square tests assuming a dominant model were performed (due to the small number of homozygotes for the rare allele). No corrections were made for multiple comparisons for two reasons. First, since the phenotypes tested (asthma, BHR, total serum IgE levels, and skin test response) are strongly associated with each other in this population, the statistical analyses do not represent independent tests. Second, we performed tests for association with phenotypes that have been observed by other investigators, both to confirm previous results and to

better characterize asthma susceptibility in our population. As described previously, all of the probands fit published criteria for an asthma diagnosis.[27] For BHR, cases were defined as probands and spouses with a $PC_{20} \leq 32$ mg/ml histamine. The control group for the analysis of both "asthma" and BHR cases were BHR-negative spouses. Individuals were considered skin-test positive if one or more skin test showed a maximum wheal diameter of > 5mm. Individuals with high total serum IgE levels were defined as having total serum IgE > 100 IU/ml, since this value best distinguished individuals with high versus low levels after examining the overall frequency distribution for the group.[5,6] Total serum IgE was also analyzed as a quantitative trait following logarithm-transformation to approximate a normal distribution.

The linkage disequilibrium test between pairs of SNPs was based on an exact test assuming multinominal probability of the multilocus genotype, conditional on the single-locus genotype.[31] A Monte Carlo simulation was used to assess the significance, by permuting the single-locus genotypes among individuals in the sample to simulate the null distribution. The empirical p-values of the LD for each pair of SNPs was based on 10,000 replicate samples.

RESULTS

Characteristics of Population Sample

As illustrated in Table 2, the probands and spouses are of similar ages (mean = 52 and 51 years, respectively). All spouses are included in the table but only BHR-negative spouses ($PC_{20} > 32$ mg/ml histamine) were used for comparison with the probands for the asthma and hyperresponsiveness phenotypes. All probands and BHR (+) spouses were included in the BHR (+) group for the BHR comparison (with BHR (-) spouses). All probands were BHR (+) at the time of initial testing. One hundred and seventy-one of 184 were retested since 13 had an FEV_1 that was too low to be retested safely ($FEV_1 < 40\%$ predicted); 10% of those retested were no longer BHR (+). A high proportion of the probands (40.3%) are very hyperresponsive ($PC_{20} <$ 2 mg/ml). Although the probands were not selected for atopy, 80.9% had ≥ 1 positive skin-test compared with 29.9% of the spouses. The degree of overlap between the asthma and atopy phenotypes in this population is shown in Figure 1.

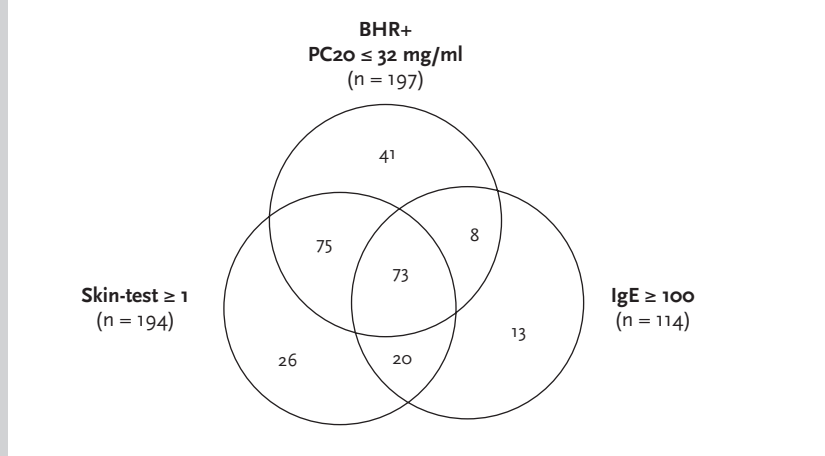
Resequencing of the IL13 gene to identify SNPs

The human IL13 gene is composed of 4 exons spanning 2.938 kb of genomic DNA .[32] SNPs in the IL13 gene were identified by resequencing genomic DNA from 20 probands and 20 controls (spouses) from the Dutch families. In addition to exons 1 to 4, over 2 kb of the 5'-flanking region and the first intron were resequenced because potential recognition sites for a number of transcription factors, interferon-inducible elements and enhancer elements have been localized to these regions .[33]

Table 2. Clinical Characteristics of Dutch Probands and Spouse

IL13 DATA	Probands	Spouses
Sex, M:F	114:70	70:114
Age, mean	52.1	51
SD	8.3	8.9
range	37-76	34-76
Total IgE, IU/ml (geometric mean)	93.0	26.5
% ≥ 100 IU/ml IgE	44.8	20.1
Skintest Response		
% with ≥ 1 positive skintest	80.9	29.9
% with ≥ 3 positive skintest	59.0	13.6
FEV_1 % Predicted Pre-Medication (mean)	69.1	98.7
FEV_1/FVC ratio (mean)	59.1	77.0
Reversibility (n)	181	184
%, ≥ 12% (baseline), (n)	66.3 (120)	12.5 (23)
%, ≥ 9% (predicted), (n)	63.5 (115)	16.8 (31)
Airway Obstruction		
% FEV_1/FVC ≤ 70% and FEV_1 ≤ 75%	52.7	2.2
PC_{20} (N)	171	183
PC_{20} > 32 mg/ml, %	9.9	75.4
PC_{20} ≤ 32 mg/ml, %	90	24.6
PC_{20} ≤ 16 mg/ml, %	81.2	16.4
PC_{20} ≤ 8 mg/ml, %	63.1	11.5
PC_{20} ≤ 4 mg/ml, %	55.5	6
PC_{20} ≤ 2 mg/ml, %	40.3	2.7
PC_{20} ≤ 1 mg/ml, %	22.8	0.0

Figure 1. Relationship of bronchial hyperrespnsiveness, skin-test response and total serum IgE levels in Dutch probands and spouses genotyped for IL13 polymorphisms.



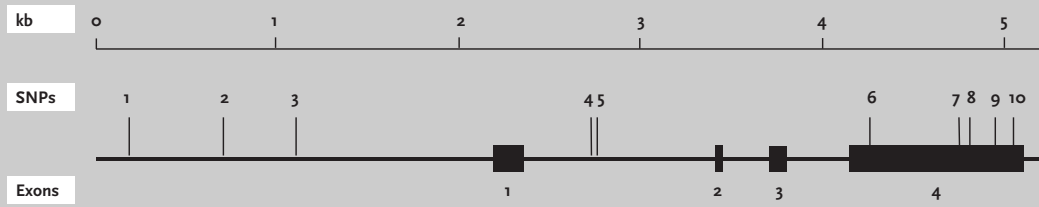


Figure 2 SNPs detected in IL13 gene

SNP	Nucleotide Position	Position Relative to +1 of ORF	Location	Allotype	Affected (n=20)	Unaffected (n=20)
1	175	-2039	5'-Promoter	CC	13	16
				CT	6	4
				TT	1	0
2	704	-1510†	5'-Promoter	AA	13	16
				AC	6	4
				CC	1	0
3	1103	-1111†	5'-Promoter	CC	15	16
				CT	4	4
				TT	1	0
4	2728	-	Intron 1	CC	16	14
				CA	2	5
				AA	1	1
5	2755	-	Intron 1	GG	17	16
				GC	2	4
				CC	0	0
6	4257	+389	Exon 4 (Arg130Gln)‡	GG	9	14
				GA	11	6
				AA	0	0
7	4738	+870	Exon 4 (3'-UTR)	GG	10	14
				GA	10	6
				AA	0	0
8	4793	+925	Exon 4 (3'-UTR)	CC	10	14
				CA	10	6
				AA	0	0
9	4962	+1094	Exon 4 (3'-UTR)	CC	10	13
				CT	10	6
				TT	0	0
10	5054	+1186	Exon 4 (3'-UTR)	TT	20	18
				TG	0	1

Figure 2. continued

SNPs Detected by resequencing of the IL13 Gene in this Dutch population. Eighteen overlapping PCR-amplified DNA fragments covering 2.214 kb of genomic DNA sequence 5' to +1 of the ORF, exons 1 to 4 (including intron-exon boundaries) and intron 1 of the IL13 gene were cycle sequenced in 20 affecteds (probands) and 20 unaffected individuals (spouses) as described in Materials and Methods. The allotype for each SNP was determined by visual inspection of the sequence traces. Top: Graphical representation of SNP locations within the IL13 gene. Bottom: Location of IL13 SNPs with allotype numbers and affection status of sequenced individuals.

* GenBank accession No. U31120 was used as the reference sequence. The coordinates for exons 1 thru 4 are nucleotides 2158-2345, 3403-3456, 3709-3813 and 4160-5095, respectively.
¶ 39 patients genotyped.
† The position of SNPs 2 and 3 relative to +1 differs between this report and Graves et al (2000) because GenBank accession number AC004039 was used as the reference sequence by the latter. The differences between U31120 and AC004039 are due to C insertions at positions 49963 and 49578 in AC004039 which are not present in U31120 (corresponding to positions 752-753 and 1136-1137, respectively).
‡ Corresponds to the Gln110Arg of Heinzmann et al. (2000) who use the amino acid coordinates of the mature protein (cytokine-web; http://www.psynix.co.uk/cytweb/targets/index.html)
UTR: untranslated region

Ten SNPs were identified by resequencing (Figure 2), with only one (SNP 6 located in exon 4) leading to a predicted amino acid change in the IL13 protein (glutamine for arginine amino acid substitution; Arg130Gln). Six of the SNPs have previously been identified in other population groups: SNP 3 (5'-promoter [14,16,34]); SNP 6 (exon 4[15-17]); SNP 2 (5'-promoter [16]); and SNPs 7, 8 and 9 (exon 4, 3'-untranslated region;[16]) SNPs 1 (5'-promoter), 4 and 5 (intron 1) and 10 (exon 4, 3'-untranslated region) are novel.

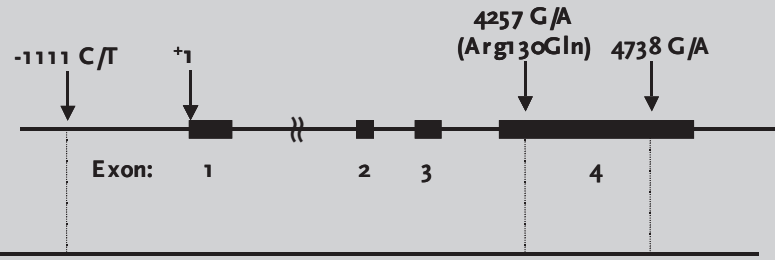
Association Analysis of IL13 with asthma and atopy phenotypes

One hundred and eighty four Dutch probands and spouses were genotyped and analyzed with SNPs 3, 6 and 7. These SNPs were located in the promoter region, exon four, and the 3' UTR (Figure 3). All SNPs were in Hardy-Weinberg equilibrium and the allele frequencies for each SNP are reported in Table 3. These frequencies are similar to those reported in other populations from the United States, Netherlands, and Germany [16], but differ from those from the United Kingdom and Japan .[17] Significant linkage disequilibrium was observed between the –1111 promoter and the 3' UTR SNP ($p<10^{-4}$) and between the Arg130Gln and 3' SNP ($p=10^{-4}$), but not between the –1111 promoter and the Arg130Gln.

Table 3. Allele Frequency in Dutch Proband/Spouse Population

	allele 1	allele 2
5' Promoter (SNP 3)	0.79	0.21
Arg130Gln (SNP 6)	0.75	0.25
3' UTR (SNP 7)	0.75	0.25

Figure 3 Location of SNPs genotyped in IL13 gene



This report:	Asthma/ ST†	Asthma/ ST†
Van der Pouw Kraan et al.:	Allergic Asthma	
Heinzmann et al.:		Asthma/ Allergic Asthma
Graves et al.:		Total serum IgE
Liu et al.:		Total serum IgE atopic dermatitis

†Skin test sensitivity

Schematic representation of the IL13 gene showing the genomic structure (boxes represent the 4 exons) and the location of SNPs genotyped in this study and others. Phenotypes with a significant association ($p<0.05$) are shown in the table below, aligned with the associated polymorphism.

The results of this study reveal significant associations between specific asthma and atopy phenotypes for the 5′ promoter and 3′ UTR SNPs (Table 4) The most significant associations were observed with the promoter polymorphism and the presence of both asthma and skin-test sensitivity (≥ 1 positive skin-test) to common aeroallergens. The promoter "T" allele was significantly more prevalent in the cases with asthma than in controls (24% versus 14%, p=0.004). This association was also present to a lesser degree in individuals with one or more positive skin-test (25% versus 17%) (p=0.01). When compared by genotype, the association with both asthma and allergy phenotypes was highly significant. TT homozygotes were much more common in cases than controls with regard to asthma (p=0.008), BHR (p=0.007), and asthma and skin-test sensitivity combined (p=0.006). While we did not observe a significant association of IL13 polymorphisms with IgE levels, there was evidence of higher total serum IgE levels with the rare allele of the promoter polymorphism (p=0.089; Table 5). When stratified by skin-test positive and skin-test negative individuals, we did not observe an association with total serum IgE levels in skin-test negative individuals, as previously reported with the Arg130Gln polymorphism.[16] Lower levels of significance were also observed with the 3′ UTR SNP (Table 4).

Table 4 Association between Asthma and Atopy Phenotypes and IL13 Polymorphisms in a Dutch Asthma Population

		Promoter			Arg130Gln			3'UTR		
		C	T	p-value	G	A	p-value	G	A	p-value
Asthma	Cases	261	81	**0.005**	230	74	n.s.	234	92	**0.043**
	Controls	204	34		178	62		184	48	
BHR	Cases	329	103	**0.003**	282	90	n.s.	294	110	0.066
	Controls	204	34		178	62		184	48	
Skin Test	Cases	289	89	**0.029**	240	84	n.s.	254	98	**0.046**
	Control	245	49		221	69		226	60	

†**Genotype**

		Promoter				Arg130Gln				3'UTR			
		CC	CT	TT	p-value	GG	GA	AA	p-value	GG	GA	AA	p-value
Asthma	Cases	99	63	9	**0.008**	89	52	11	n.s.	86	62	15	**0.02**
	Controls	87	30	2		67	44	9		77	30	9	
BHR	Cases	126	77	13	**0.007**	111	60	15	n.s.	109	76	17	n.s.
	Controls	87	30	2		67	44	9		77	30	9	
Skin Test	Cases	111	67	11	n.s.	89	62	11	n.s.	93	68	15	**0.02**
	Controls	102	41	4		89	43	13		94	38	11	
Asthma *and*	Cases	79	52	8	**0.006**	69	46	8	n.s.	66	53	13	**0.009**
Pos. skin-test	Controls	87	30	2		67	44	9		77	30	9	

*Analysis was performed using the number of each allele in cases and controls

†Analysis was performed using a dominant model for the genotypes in cases and controls

Table 5 Mean IgE levels for each genotype

Promoter	*Number*	*Mean Log [IgE] (± SD)*	*Geometric Mean total serum IgE (IU/ml)*	*p-value*
CC	213	1.63 (± 0.73)	42.66	n.s.
CT	108	1.79 (± 0.71)	61.66	
TT	15	1.87 (± 0.74)	74.13	
Arg130Gln				
GG	178	1.63 (± 0.69)	42.66	n.s.
GA	105	1.81 (± 0.72)	64.57	
AA	24	1.71 (± 0.75)	51.29	
3' UTR				
GG	187	1.63 (± 0.70)	42.66	n.s.
GA	106	1.82 (± 0.74)	66.07	
AA	26	1.77 (± 0.76)	58.88	

DISCUSSION

Asthma is an inflammatory airways disease characterized by bronchial hyperresponsiveness and airways obstruction. Atopy traits, such as elevated total serum IgE levels and positive allergen skin-test responses, are also associated with this disease and may predict the development of symptomatic asthma.[1,2,4] Interleukin 13 is expressed in asthmatic airways and has an important role in the production of IgE and is therefore an excellent biologic candidate gene for the development or expression of diseases with atopy components such as asthma.

In this study of the IL13 gene, 10 SNPs were detected of which 6 have been identified previously and 4 are novel. Of the former, SNPs 3 (-1111 promoter) and 6 (Arg130Gln) have been associated with increased risk of allergic asthma[14] and higher total serum IgE levels[16] or asthma[17], respectively. In addition, SNP 3 appears to promote increased binding of nuclear proteins to the promoter region[14], whereas the amino acid change resulting from SNP 6 could affect the interaction of IL13 with IL13Rα1.[16,17] In contrast, none of the remaining 8 SNPs occur within identified regulatory elements in the IL13 gene[e.g. 33], or alter the amino acid sequence, so their functional relevance is unclear at the present time. We investigated the contribution of SNPs 3, 6 and 7 using a case-control study in a Dutch asthma population consisting of probands, selected on the basis of a diagnosis of asthma, and their unaffected spouses. One SNP was chosen in the 3' UTR (SNP 7, nt 4738) to examine the potential regulatory elements in this region. These three SNPs encompassed the entire IL13 gene so that the contribution of genetic variation could be detected. Consistent with other reports that have evaluated IL13 polymorphisms, we observed a significant association of several polymorphisms in IL13 with various atopy or asthma phenotypes.

There are several unique properties of this study. The cases and controls are the parents in the same families used to identify linkage to chromosome 5q31, allowing us to examine candidate genes within this region in linked families. The comprehensive clinical data collected in these families have allowed us to examine multiple clinical phenotypes associated with both asthma and atopy. And finally, because of the study design, the controls were similar in regard to age and overall environmental exposures (allergens).
Both IL4 and IL13 can elevate baseline IgE levels. However, while an essential role for IL4 in the induction of asthma has been proposed, murine models have demonstrated the critical nature of IL13 independent of IL4. In a murine model of allergic airways hyperresponsiveness, blockade of IL13 reversed many of the characteristics found in allergic asthma such as airway hyperresponsiveness, eosinophil infiltration, and mucous production.[35] Furthermore, the effects of IL13 were shown to be mediated by a pathway dependent on the IL4Rα receptor.[35] Transgenic mice expressing IL13 specifically in the lungs exhibited increased BHR, bronchial eosinophilia, and increased mucous production.[36]
Using a mouse model for asthma, Symula et al.[37] were able to demonstrate the effects of quantitatively changing mouse IL4 and IL13 gene expression using transgenic mice constructed with human YACs from the chromosome 5q3 region. Surprisingly, the transgenic mice had significantly lower total IgE levels due to decreased endogenous gene expression, which influenced the development of Th2 cells. (Human IL4 and IL13 appear to have minimal activity in mice). Furthermore, when a BAC containing mouse IL4 and IL13 genes was transfected into the mice, a significant increase in IgE levels, BHR, and asthma was observed.[37]

It has been suggested that IL13 may be an important regulatory cytokine in the pathogenesis of asthma, while IL4Rα, which is required for the functioning of both IL4 and IL13, contributes primarily to atopy.[38] Several IL4Rα polymorphisms have been associated with a higher risk of atopy[19,21], atopic asthma[15], and variation in IgE levels.[21] In addition, specific alleles of these variants were shown to modulate the activity of IL4Rα.[19-21] In a recent study by Ober et al.[22] eight polymorphisms were studied in both inbred and outbred populations. Significant evidence for an association between these variants and the resulting haplotypes were observed for asthma and atopy. These studies indicate that biologic functions related to these disorders may involve this receptor.

The most significant associations with IL13 were identified in individuals with a diagnosis of asthma (i.e., the original probands in this study) and in individuals with the BHR phenotype (including affected spouses) (Table 4). The study by Heinzmann et al.[17] also supports this role of IL13 in asthma. In both the Japanese and the British populations, the most significant associations were observed between IL13 (Gln110Arg) and atopy and nonatopy asthma. There was no evidence of an association with this polymorphism and total serum IgE levels. In the Japanese population, strong asso-

ciation was observed with the Ile50Val IL4Rα polymorphism and IgE levels, both total serum and allergen-specific ($p<0.0001$). The coexistence of BHR and atopy characteristics (e.g., Figure 1) makes it difficult to discern the exact roles of IL4Rα and IL13. However, the interaction of these two genes as important components of IgE-mediated inflammatory responses supports the role of these cytokine pathways in the development or expression of atopy conditions and asthma.

Association analyses with specific polymorphisms in IL13 have produced varied results (Figure 3).[14-17] This may be due, in part, to the fact that each study is based on population samples that were ascertained differently. For example, the previous studies focused on recruitment of patients with allergic asthma[14,17], atopy[15], or random ascertainment from longitudinal and cross-sectional groups.[16] In our study design, we ascertained families based on asthma, but are also able to examine both asthma and atopy phenotypes. In addition, these families showed evidence for linkage to the region of chromosome 5q where IL13 is located.[5-7]

Another potential cause of differences in the results of these studies is that they were performed in different population groups. As linkage disequilibrium varies between populations, this would suggest one of two possibilities. First, it is possible that different polymorphisms or haplotypes within the IL13 gene contribute to the allergy phenotype in each population. Therefore, each analysis may be identifying the specific allele or haplotype responsible for the phenotype in that specific population. This suggests that several of the polymorphisms identified are capable of significantly altering the function of IL13, resulting in a predisposition to atopy, and reported differences are primarily due to the founder allele in that specific population. A second explanation is that an additional, unidentified sequence variant is responsible for the phenotype, and the level of detection (i.e. significance of the association) is dependent on the degree of linkage disequilibrium in that population for this region of chromosome 5q. In this case, the true susceptibility allele would have to be a fairly distant enhancer or promoter element, since IL13 resequencing studies have most likely identified all of the common variations within or near the gene (this report).[14,16] One candidate for such a distal element would be the conserved noncoding sequence (CNS-1) recently identified between IL13 and IL4.[39] This sequence is approximately 3kb from the 3' end of IL13 and 10kb from the 5' end of IL4, is highly conserved between mice, humans, cows, dogs, and rabbits (~80% identity), and was shown to specifically regulate IL4, IL13, and IL5 in human YAC transgenic mice. This region should be investigated to determine if polymorphisms are present that may influence susceptibility or expression of atopy or asthma. A variation in CNS-1 may explain the different results from the IL13 studies, and may also explain the conflicting results with a promoter polymorphism in IL4[13], since various degrees of linkage disequilibrium with CNS-1 and the IL13 promoter between populations may contribute to differences in reported associations.

Asthma and allergy are common diseases caused by an intricate interaction of genetic susceptibility and environmental exposure. This association study, as well as those reported by others, has implicated IL13 as a major component in the expression of these conditions. Functional studies examining the roles that these polymorphisms have on the activity or expression levels of IL13 and, perhaps more importantly, the downstream responses to these changes will provide valuable insight into the overall mechanisms that cause susceptibility to asthma and atopy. As we define specific genes that are associated with allergy and asthma phenotypes, patterns are beginning to develop that may be useful in delineating important biologic pathways, leading to a better understanding of gene-gene and gene-phenotype relationships in asthma and allergy. The importance of IL13 and its functional and genetic interactions with IL4 and IL4R in the development and expression asthma and atopy support this approach that combines molecular genetic techniques with clinical studies.

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Chapter 10 | Association of IL4Rα polmorphisms with atopy and asthma and gene-gene interaction with IL13 in an asthmatic Dutch population

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ABSTRACT

Asthma is a common respiratory disease that is characterized by variable airways obstruction caused by acute and chronic bronchial inflammation. Clinical findings in asthma include bronchial hyperresponsiveness and allergic responses, demonstrated by elevated total serum IgE levels and positive skin tests to common allergens. These closely associated clinical phenotypes have been shown to have a strong genetic component. Binding of IL4 to the IL4 receptor (IL4R) induces the initial response for Th2 polarization. IL4 and IL13 are both produced by Th2 cells and are capable of inducing isotype class-switching of B-cells to produce IgE after allergen exposure. These cytokines also share a common receptor component, IL4Rα, which is a potential biological candidate gene for asthma and atopy. We have investigated five IL4Rα single-nucleotide polymorphisms in a well-characterized population of Dutch families ascertained through a proband with asthma that was initially studied 25 to 35 years ago. Using the probands and their spouses from this population in a case-control study design, we observed significant associations of atopy and asthma related phenotypes with several IL4Rα of the polymorphisms genotyped within the gene. The most significant association was observed with S478P, which was associated with high IgE levels ($p = 0.0007$). In addition, a significant gene-gene interaction was detected between the S478P variation in IL4Rα (significantly associated with high IgE levels) and the –1111 promoter variation in IL13 (significantly associated with hyperresponsiveness). Individuals with the risk genotype for both of these genes were at almost five times higher risk for the development of asthma compared to individuals with both non-risk genotypes ($p = 0.0004$). These data suggest that variations in IL4Rα contribute to elevated total serum IgE levels, and interaction between IL4Rα and IL13 markedly increases an individual's susceptibility to asthma in this Dutch population.

INTRODUCTION

Asthma is an increasingly common inflammatory airways disease characterized by bronchial hyperresponsiveness (BHR) and reversible, intermittent airways obstruction. Multiple studies have demonstrated a strong genetic component to this disease.[1,2] Specifically, BHR and atopic responses such as elevated total serum IgE levels, typical findings in asthma, have been used to identify regions of the genome that may contain genes that play a role in the pathogenesis of asthma and allergy.[3-6]

The interleukin 4 receptor (IL4R) is a key component in the induction of the Th2 phenotype and IgE production. Binding of IL4 to the IL4R initiates B-cell switching from IgG to IgE production after allergen exposure. Antigen-presenting cells stimulate production and secretion of IL4 from T-cells, leading to a Th2 cell phenotype and the subsequent switch to IgE synthesis.[7] A further role of IL4 in the pathogenesis of asthma has been indicated from actively sensitized IL4 knockout mice. Neither specific IgE induction nor bronchial hyperresponsiveness were detected in these mice[8,9] suggesting a critical role for the IL4/IL4R pathway in these phenotypes. The pleiotrophic effects of IL4 are mediated through the IL4R, which is comprised of the high affinity α subunit, and either the common γ subunit or the IL13 receptor α subunit. The IL13 receptor consists of one IL4Rα subunit and either a low-affinity IL13Rα1[10] or a high-affinity IL13Rα2 subunit.[11] The complete receptor for IL4 is composed of one IL4Rα subunit and an IL4Rγ subunit. Therefore, it is possible that different polymorphisms in these receptors, as well as in the IL4 and IL13 cytokines, contribute to the complex regulation of atopy or asthma phenotypes. IL13 also contributes to the maintenance of the Th2 profile that leads to elevated baseline IgE levels. In fact, murine models have demonstrated the critical nature of IL13 independent of IL4.[12]

Since increased levels of total serum IgE have been strongly correlated with asthma and BHR[4,13,14], and mouse models suggest that IL4 and IL13 may modulate atopy and asthma related phenotypes[15], IL4, IL13, and IL4Rα are excellent candidate genes for these conditions. At least thirteen polymorphisms have been reported within the IL4Rα gene.[16-18] The I50V, S478P, and Q551R variants have been associated with a higher risk of atopy[17,19], atopic asthma[20], and variation in IgE levels.[17] In addition, specific alleles of these variants were shown to modulate the activity of IL4Rα.[19,21] In a recent study by Ober and coworkers[18], eight polymorphisms in IL4Rα (seven in exon 12) were studied in both inbred and outbred populations. Significant evidence for an association between several of these variants and the resulting haplotypes were observed for asthma and atopy. We eva luated five polymorphisms (four in exon 12) within IL4Rα in a Dutch po pulation ascertained through a proband with asthma to determine the importance of these variants for susceptibility and expression of asthma and

atopy in this population. Given the biological role of IL4Rα, our primary hypothesis was to test for differences in total serum IgE levels between IL4Rα genotypes, and then to investigate associated phenotypes. Since we have previously found a significant association between IL13 polymorphisms and BHR (Howard et al., submitted) and between IL4Rα and total serum IgE levels in the current investigation, we performed a gene-gene interaction analysis for these two candidate genes and asthma susceptibility.

MATERIALS AND METHODS

Population

This population has been described in detail previously.[3,22] Ascertainment was based on a formal diagnosis of asthma in a clinical setting. The probands with asthma were originally characterized between 1962-1975. Between 1990 and 1998, 200 probands with asthma (together with their spouses, children and available grandchildren) were restudied. Briefly, all individuals underwent spirometry, reversibility to 800 mg albuterol, and bronchial responsiveness testing to histamine using a 30 second inhalation protocol previously described.[22;23] For atopy, adult subjects had intracutaneous skin testing with 16 common aeroallergens and total serum IgE le vels were measured. In the first 92 families, total serum IgE levels were measured by solid-phase immunoassay (Pharmacia Diagnostics, Uppsala, Sweden). Duplicate measurements were made and the mean for each subject was used. If the duplicate samples differed by more than five percent the test was repeated. In the second set of 108 families, total IgE levels were measured by enzyme linked fluorescence assay (Mini Vidas, Biomerieux, Inc.). Although entire families were ascertained for linkage studies, the probands and spouses represent an appropriate cohort for this case-control association study. Probands and spouses have a similar age, removing the confounding effect of age on changes in BHR, IgE and atopy. This study was approved by the Medical Ethics Committee at the University of Groningen. All subjects gave written informed consent.

Molecular Methods

DNA was isolated using standard techniques. For the I50V, E375A, and C406R polymorphisms, PCR was carried out using standard conditions and an annealing temperature of 60°C (I50V), and 68°C (E375A, C406R), and the previously described primers for I50V[20], and 5′-CAGCATGGTGC-CCAGTGGAG-3′ and 5′-CTTGGGAACTCATCCCAGGGC -3′ for E375A and C406R. The E375A and C406R polymorphisms were contained within the same 334 bp PCR product and digested with the restriction enzymes Cac8I (E375A) or Tsp45I (C406R) to distinguish the alleles. The I50V PCR products were digested with the restriction enzyme MslII.

The S478P and Q551R polymorphisms were genotyped using a novel variation of allele-specific PCR that utilizes fluorescent dye and automated

sequencer technology (FAS-PCR).[24] Allele-specific primers were designed for both polymorphisms using the known IL4R sequence (GenBank accession no. X52425) and previously published data.[17,19] Two allele-specific forward primers were designed with different fluorescent labels and an addition of two nucleotides to one. The S478-specific primer was (tet) 5′-TGCTTACCGCAGCTTCAGCAACT-3′ and the P478-specific primer was (fam) 5′-CTTACCGCAGCTTCAGCAACC-3′. The common reverse primer was 5′-TTTCTGGCTCAGGTTGGGGC-3′. The forward primer specific for the Q551 allele was (tet) 5′-GGCCCCCACCAGTGGCTATCA-3′ and the primer specific for the R551 allele was (fam) 5′-CCCCCACCAGTGGCTATCG-3′. The same reverse primer, 5′-CCAGTCCAAAGGTGAACAAGGGG-3′, was used to detect each of the allele-specific products. One-fourth of the Q551 specific primer was used to compensate for the increased intensity of this PCR amplified product. Fragments were separated and analyzed with ABI 377 DNA Sequencers. The IL13 -1111 promoter SNP was also genotyped using a PCR-RFLP assay using the primers 5′-ATGCCTTGTGAGGAGGGTCAC-3′ and 5′-CCAGTCTCTGCAGGATCAACC-3′. PCR products were digested with NheI (New England Biolabs) and the alleles resolved by electrophoresis on a 2% agarose gel.

Genetic Analysis

Analysis was performed for the following phenotypes: total serum IgE levels, skin test responsiveness to common allergens, asthma, and BHR. As described previously, all of the probands fit published criteria for a diagnosis of asthma.[22] Total serum IgE was analyzed as a qualitative and quantitative trait. As a qualitative trait, cases were defined as having total serum IgE > 100 IU/ml; this value best distinguished them from those not affected after examining the overall frequency distribution.[4,5] As a quantitative trait, IgE was logarithm-transformed to approximate a normal distribution. Differences between groups were tested with ANOVA, t-test, and multiple regression. Individuals were considered responsive to an allergen skin test if one or more test showed a mean wheal diameter of > 5mm. For BHR, cases were defined as original probands and spouses with a PC20≤ 32 mg/ml histamine. The control group for both BHR-positive and asthma cases was comprised of BHR-negative spouses (PC20 > 32 mg/ml). Each of the biallelic polymorphisms was analyzed by comparing differences of genotype frequencies between cases and controls. Chi-square tests assu ming a dominant model were performed, due to the small number of homozygotes for the rare allele. For the interaction analysis, the individuals with the non-risk genotypes at each gene polymorphism, based on this and previous results in our study population, were compared with individuals carrying either, or both, risk genotypes.

The linkage disequilibrium test between pairs of SNPs was based on an exact test assuming multinominal probability of the multi-locus genotype, conditional on the single-locus genotype.[25] A Monte Carlo simulation was

used to assess the significance, by permuting the single-locus genotypes among individuals in the sample to simulate the null distribution. The empirical p-values of the LD for each pair of SNPs were based on 10,000 replicate samples.

Table 1. Clinical characteristics of Dutch proband/spouse population*

	Probands	Spouses
Sex, M:F	124:76	76:125
Age, mean	52.1	51.0
SD	8.4	9.2
IgE		
Total IgE, IU (geometric mean)	93.0	26.2
% ≥ 100 IU/ml	72.5	15.4
Skintests		
% with ≥1 positive skintest	81.9	31.0
FEV_1		
% Predicted pre-medication (mean)	69.6	98.4
% Predicted post-medication (mean)	82.4	103.9
Reversibility		
%, ≥ 15% (baseline)	59.4	6.5
%, ≥ 9% (predicted)	62.9	18.9
Airway obstruction		
% FEV_1/VC ≤ 70% and FEV_1 ≤ 75%	51.0	3.0
BHR †		
PC_{20} ≤ 32 mg/ml, %	88.2	25.6

* Total sample population consisted of 200 probands and 201 spouses. Different numbers for the SNPs in the following tables are due to missing genotype data.
† Thirty probands were not retested due to an FEV_1 that was too low to be tested safely (FEV_1 ≤ 40% predicted).

RESULTS

Clinical characteristics of population

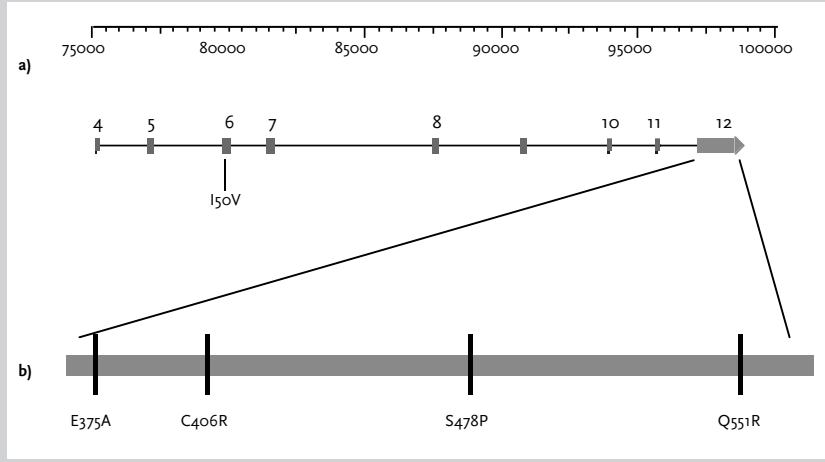
As illustrated in Table 1, the probands and spouses are of similar ages (mean = 52.1 and 51.0 years, respectively). All spouses are included in the table but only BHR-negative spouses (PC_{20} > 32 mg/ml histamine) were used for comparison with the probands for the asthma and BHR phenotypes. All probands and BHR-positive spouses were included in the BHR-positive group for the BHR comparison (with BHR-negative spouses). All

probands were BHR-positive at the time of initial testing. Only 170 of 200 probands were retested, since 30 had an FEV_1 that was too low to be retes ted safely (FEV_1 < 40% predicted); 11.1% of those retested were no longer BHR-positive. A high proportion of the probands (42.9%) were very hyper-responsive to histamine (PC_{20} < 2 mg/ml). Although the probands were not selected for atopy, 81.9% had > 1 positive skin test compared with 31.0% of the spouses.

Association analysis of IL4Rα polymorphisms

The IL4Rα gene contains 12 exons and spans a genomic distance of approximately 51 kb.[17] All of the polymorphisms studied in this report, except I50V, fall within exon 12 of the gene and are therefore within a 528 bp interval (Figure 1). The I50V polymorphism encodes the extracellular portion of the receptor molecule and is located approximately 20 kb upstream from this region.

Figure 1 Genomic structure of the IL4Rα gene.



Genomic structure of the IL4Rα gene. a) The top line represents the genomic sequence of the IL4Rα region of chromosome 16. The bottom line indicates the locations of the exons (boxes) and the introns of IL4Rα aligned to the BAC sequence. b) Four SNPs examined in this association study from exon 12 of IL4Rα.

The probands and spouses from the 200 Dutch families were analyzed with the four exon 12 polymorphisms. Since there was no evidence of association with this SNP and any of the tested phenotypes, the I50V polymorphism was only genotyped in an initial set of 109 probands and 111 spouses. The allele frequencies from this Dutch population were V50 = 0.47, A375 = 0.12, R406 = 0.12, P478 = 0.16, and R551 = 0.20. Each polymorphism was in Hardy-Weinberg equilibrium for the entire population evaluated in this study.

Table 2. Association between Log(IgE) levels and IL4Rα Polymorphisms in Dutch Families.

Polymorphisms	N	Geometric Mean (IU/ml)	Log (Mean ± SD)	P-value*
I50V				
II	65	34.7	1.54 ± 0.66	
IV and VV	155	53.7	1.73 ± 0.71	0.07
E375A				
EE	258	56.2	1.75 ± 0.74	
EA and AA	75	34.7	1.54 ± 0.63	0.02
C406R				
CC	259	55.0	1.74 ± 0.74	
CR and RR	74	32.4	1.51 ± 0.65	0.01
S478P				
SS	204	64.6	1.81 ± 0.72	
SP and PP	81	31.6	1.50 ± 0.6	0.0007
Q551R				
QQ	226	53.7	1.73 ± 0.73	
QR and RR	113	38.0	1.58 ± 0.69	0.06

* Adjusted for age and sex, and comparing the genotypes '1/1' vs. '1/2 and 2/2'. Chi-square tests were performed using log (IgE) values, since the geometric means were not normally distributed.

A significant association was observed between E375A, C406R, and S478P and increased levels of total serum IgE ($p = 0.0007$-0.02; Table 2). S478P was also associated with one or more positive skin tests ($p = 0.03$; Table 3). In each case the common allele (E375, C406, and S478) was associated with the phenotype. Because of the observed relationship between BHR and elevated total IgE levels[13,14], BHR and asthma were also examined with each of the polymorphisms (Table 3). S478P was the only variation associated with this phenotype ($p = 0.02$ - 0.04; Table 3). More of the BHR-positive indivi duals ($PC20 \leq 32$ mg/ml) were homozygous for the S478 allele than BHR-negative individuals.

Linkage disequilibrium between polymorphisms

Since the four polymorphisms in exon 12 of IL4Rα were only 528 bp apart, we tested for linkage disequilibrium (LD) between the SNPs. In this homogenous population, significant LD between E375A, C406R, S478P, and Q551R was observed ($p<10^{-5}$). The most common haplotype was E375, C406, S478, and Q551. In contrast, LD was not observed between these four polymorphisms and the I50V polymorphism. Due to the degree of LD between the four exon 12 SNPs, construction of haplotypes did not improve the evidence for association with the S478P polymorphism individually (data not shown).

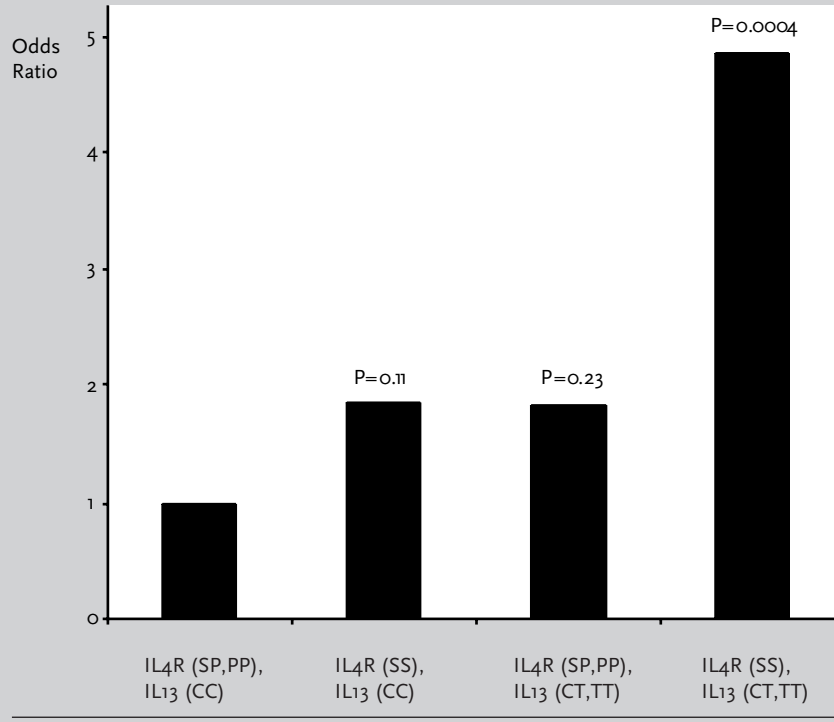
Table 3. Association between asthma and allergy phenotypes and IL4R α Polymorphisms in Dutch families

	Asthma (%)		BHR (%)		Skin Test (%)	
SNPs	Probands	BHR-neg. spouses	$PC_{20} \leq 32$mg/ml	$PC_{20} > 32$mg/ml	≥1	0
I50V	*n=109*	*n=87*	*n=126*	*n=94*	*n=116*	*n=105*
II	0.28	0.29	0.29	0.30	0.26	0.33
IV	0.49	0.48	0.46	0.49	0.48	0.47
VV	0.24	0.23	0.25	0.21	0.26	0.20
	p = 0.85		p = 0.79		p = 0.16	
E375A	*n=150*	*n=112*	*n=178*	*n=124*	*n=166*	*n=137*
EE	0.81	0.71	0.81	0.73	0.82	0.72
EA	0.16	0.28	0.16	0.26	0.16	0.26
AA	0.03	0.01	0.03	0.01	0.02	0.01
	p = 0.06		p = 0.16		p = 0.09	
C406R	*n=148*	*n=108*	*n=175*	*n=120*	*n=162*	*n=134*
CC	0.82	0.71	0.82	0.73	0.82	0.73
CR	0.16	0.28	0.16	0.26	0.16	0.25
RR	0.03	0.01	0.02	0.01	0.02	0.01
	p = 0.05		p = 0.09		**p = 0.05**	

Table 3. Association between asthma and allergy phenotypes and IL4R αPolymorphisms in Dutch families (continued)

S478P	*n=144*		*n=103*	*n=171*		*n=114*	*n=160*		*n=126*
SS	0.77		0.63	0.75		0.67	0.77		0.65
SP	0.20		0.33	0.22		0.30	0.20		0.31
PP	0.03		0.04	0.03		0.04	0.03		0.04
		p = 0.02			**p = 0.04**			**p = 0.03**	
Q551R	*n=151*		*n=114*	*n=181*		*n=127*	*n=169*		*n=140*
QQ	0.68		0.64	0.68		0.65	0.69		0.63
QR	0.26		0.32	0.25		0.31	0.25		0.31
RR	0.05		0.04	0.07		0.04	0.05		0.06
		p = 0.48			p = 0.64			p = 0.2	

Figure 2 Interaction of IL4Rα and IL13 Genotypes.



Bars indicate the odds ratios between the different combinations of genotypes for IL4Rα (S478P) and IL13 (-1111 C/T). The non-risk genotype for each gene was used as the reference odds ratio.

Interaction of IL4Rα and IL13 genotypes

In addition to single SNP and haplotype analysis, an interaction analysis was performed. Because of the biological relationship of IL4Rα and IL13, analysis was performed to determine if individuals with the risk genotypes for both genes were at an increased risk of developing asthma. The most significantly associated SNPs for IL4Rα (S478P with total serum IgE levels) and IL13 (–1111 C/T with BHR; Howard et al., submitted) were examined for potential gene-gene interaction. Each SNP individually was significantly associated with the asthma phenotype. An even greater interaction effect was observed in individuals with the risk genotype for both IL4Rα and IL13 (OR = 4.87, p = 0.0004). This effect was most notable in those individuals homozygous for the common allele for IL4Rα (recessive effect) and in those individuals with the rare allele for IL13 (dominant effect), consistent with the results for the individual SNPs (Table 4, Figure 2). A similar analysis was performed examining total serum IgE levels. In this case, the interaction between the two genes was significant, but less than the effect of IL4Rα S478P alone.

Table 4. Interaction of IL4Rα (S478P) and IL13 (-1111 C/T) Genes on Asthma

		Number of individuals				
IL4Rα	**IL13**	**Case**	**Control**	**OR**	**95% C.I**	**P-values**
(SP, PP)	(CC)	16	22	1		
(SS)	(CC)	62	46	1.85	0.88-3.92	0.11
(SP, PP)	(CT, TT)	16	12	1.83	0.68-4.92	0.23
(SS)	(CT, TT)	46	13	4.87	2.00-11.86	0.0004

DISCUSSION

We have observed significant association between IL4Rα SNPs and atopic phenotypes, most significantly, total serum IgE levels. In addition, gene-gene interaction was detected between IL4Rα and IL13, based on one polymorphism in each gene. Specific alleles of IL4Rα have been associated with atopy and asthma phenotypes and increased gene activity in previous stu dies. We have evaluated five polymorphisms in the IL4Rα gene in a well-characterized Dutch population ascertained through a proband with asthma for association with susceptibility to asthma and atopy and associated phenotypes. Analysis of three polymorphisms within exon 12 of IL4Rα revealed an association with increased total serum IgE levels and several phenotypes associated with asthma. No association was observed with I50V, the only polymorphism examined in the extracellular-coding portion of the gene. We have also identified a significant gene-gene interaction between IL4Rα and IL13, which has been previously shown to be highly significantly associated with the expression of hyperresponsiveness. Individuals with the risk genotype for both genes are at almost a five-fold increase of developing asthma compared to individuals with both non-risk genotypes.

Asthma is an inflammatory airways disease characterized by bronchial hyperresponsiveness and variable airways obstruction. Atopic traits such as elevated total serum IgE levels and positive skin responses are closely associated with this condition and may predict the development of symptomatic asthma.[13,14,26] Interleukin 4 and its receptor play key roles in the regulation of IgE levels and are therefore excellent candidate genes for the development or expression of atopic phenotypes associated with asthma. In addition to the most significant association between IL4Rα and higher total serum IgE levels, we also observed an increased prevalence of the homozygous S478 genotype in BHR-positive individuals (p=0.04). This observation is probably due to the strong correlation between asthma and atopy related phenotypes described by others[13], and also noted in this group of Dutch families (Table 1). The mean total serum IgE in the probands from this cohort was 93.0 IU/ml compared to 26.2 IU/ml in their spouses.

Further evidence for the importance of IL4R in atopic disorders and asthma comes from recent therapeutic reports on the use of soluble IL4R in these conditions.[27,28] These studies show that an IL4R antagonist can effect immunoglobulin synthesis and, in early clinical trials, improve respiratory function and asthma control. Our results, in addition to others identifying IL4Rα as a key component of atopy or asthma-related phenotypes, suggest that screening of this gene may identify those individuals at risk of developing asthma due to the IL4/IL13 pathway. This may lead to better treatment efficacy using appropriate therapeutic interventions, including soluble IL4Rα. In addition, it will be of interest to determine whether IL4Rα polymorphisms affect the response to an IL4R antagonist, as has been shown with other pharmacogenetic relationships.[29,30]

There is a large degree of variability in the results of association studies with IL4Rα polymorphisms. These differences can only be addressed by examining multiple polymorphisms within the gene, as was performed in this and some other studies.[17,18] For instance, two groups have reported an increase in IgE levels and association with an atopic phenotype with two different polymorphisms.[19;20] These reports also provided functional data to corroborate the observed phenotype in their patient population. A potential reason for differences in these previous studies is that only one polymorphism was analyzed; thus, the genotype for the remaining polymorphisms was unknown. For example, if the "true" susceptibility allele causing increased total serum IgE levels is the E375 allele, this allele may have been in linkage disequilibrium with the R551 allele in the original study.[19] Likewise, this allele may have been in disequilibrium with the I50 allele in the study in the Japanese population.[20] Unless the genotype at several of the polymorphic sites is known within the IL4Rα gene and a ha plotype is constructed, it may be difficult to determine which polymorphism is responsible for a given phenotype and a subsequent increased or decreased expression of an allergic phenotype.

This report did not show evidence that the I50V and the Q551R are the primary polymorphisms responsible for allergy susceptibility. Instead, our data suggest that the polymorphism associated with asthma and atopic phenotypes is either the E375A, C406R, S478P variation, a combination of these loci, or an additional yet unknown variant in linkage disequilibrium with these polymorphisms. Because of the complete linkage disequilibrium between the exon 12 polymorphisms in this population, it is impossible to determine which, if any, is the true susceptibility polymorphism without functional data. Alternatively, association studies in a population where LD does not exist between these polymorphisms may help to define the single variant, or the combination of variants that contribute to susceptibility. It is possible that a specific haplotype induces conformational changes that together increase the overall effect.[17] The data presented in this report are consistent with the recent study reporting association of the P478 and R551 haplotype with lower IgE levels.[17]

The evidence provided in this report and in previous studies suggests that IL4Rα may represent a susceptibility gene for asthma and atopy. This locus may also be responsible for modulating asthma severity. In an association study of 149 asthma patients and controls, a significant association was observed with the Q551R polymorphism and FEV_1 (percent predicted) when stratified by mild (FEV_1 >80%), moderate (FEV_1 60%-80%), or severe (FEV_1 <60%).[31] Of the severe asthmatics, 52.6% were homozygous for the R551 allele, compared with 10.5% of the mild asthmatics (p = 0.015).[31] We did not observe this association in our data with the Q551R polymorphism.

Because of the biological interaction between IL4Rα and IL13 in the development of atopy, we have assessed whether polymorphisms in these genes have an interactive effect on expression of atopy and asthma. Indeed, we have identified a significant interaction between polymorphisms in diffe rent genes controlling asthma, i.e., IL4Rα and IL13. Individuals with the risk genotype for the SNPs examined in each gene were almost five times more likely to develop asthma compared to individuals with both non-risk genotypes (Table 4, Figure 2). This effect was much more significant than the effect of either gene alone. While we also observed a significant interaction between IL4Rα and IL13 controlling total serum IgE levels (p = 0.005), this effect was much less than the association of the S478P polymorphism alone with total serum IgE levels (Table 2). This observation suggests a potential etiology, at least in some individuals, for asthma susceptibility. We have previously demonstrated that variants in IL13 contribute significantly to BHR susceptibility (p = 0.007; Howard et al, submitted), but not to total serum IgE levels. In this study, we report a significant association of variants in IL4Rα with elevated total serum IgE levels and only borderline significance with BHR and asthma. More importantly, individuals with the risk genotypes for both of these genes are much more susceptible to asthma, which is a composite of both elevated total serum IgE levels and BHR. Individually, variants of IL4Rα and IL13 may lead to susceptibility of elevated total serum IgE levels and BHR, respectively. Combined, however, they lead to a marked increased risk of developing asthma.
This observation suggests that there is a genetic, as well as biological, interaction between the IL4Rα and IL13 gene products. The IL13 promoter polymorphism most likely effects transcriptional regulation of the gene, whereas the S478P variation of IL4Rα has been suggested to alter the conformation of the receptor protein and possibly modify downstream signaling.[17] Increased amounts of IL13 cytokine may enhance the effect of the altered IL4R receptor complex, intensifying the downstream response. Functional assays are necessary to support this hypothesis.

Asthma and allergy are conditions with complex immunologic, physiologic, and inflammatory etiologies. This is supported by biologic studies in animal models and human studies as well as the results of numerous genome screens conducted in various populations. These genome screens

revealed that different chromosomal loci contribute to asthma or allergy phenotypes in different ethnic groups.[e.g.4,32-36] It is apparent that IL4Rα contributes to atopic phenotypes in at least some populations based on this and previous studies.[18-21] In addition, SNPs in IL13 have been associa ted with increased risk of allergic asthma[37], higher total serum IgE levels[38] [39] and asthma[40] (Howard et al., submitted). Functional studies examining the individual roles of IL4 and IL13 gene products as well as their biologic interactions and the resulting downstream responses will provide valuable insight into the overall mechanisms that cause susceptibility to asthma and atopy. In summary, this study supports observations in other populations suggesting that polymorphisms in IL4Rα are associated with asthma and atopy related phenotypes. Increased total serum IgE levels were signi ficantly associated with three of the five polymorphisms tested within the gene. An additional observation was a significant interaction between polymorphisms in IL4Rα and IL13 that contributes to asthma susceptibility.

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Chapter 11 ß2 adrenoceptor promoter polymorphisms: extended haplotypes and functional effects in peripheral blood mononuclear cells.

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Abstract:

Background

The β_2-adrenoceptor and its 5′ untranslated region contain a number of genetic variants. The aim of this study was to investigate the potential for genetic variation at this locus to influence the expression of β_2-adrenoceptors on circulating peripheral blood mononuclear cells (PBMCs).

Methods. Genotype was determined in 96 individuals with asthma for 4 polymorphisms at the β_2-adrenoceptor locus. β_2-adrenoceptor binding and cyclic AMP responses to isoprenaline in PBMCs were determined and the relationship between genotype/haplotype and β_2-adrenoceptor expression and response to isoprenaline examined.

Results. This study demonstrated that β_2-adrenoceptor promoter polymorphisms are common in the Caucasian population. Strong linkage disequilibrium exists across this locus resulting in the occurrence of several common haplotypes. No single polymorphism nor haplotype was correlated with the level of β_2-adrenoceptor expression or cyclic AMP responses to isoprenaline in vitro.

Conclusion. We conclude that β_2-adrenoceptor polymorphisms when considered in isolation or by extended haplotypes do not determine the basal level of expression or coupling of β_2-adrenoceptors in PBMCs obtained from asthmatic subjects.

Introduction

β_2-Adrenoceptor agonists remain the mainstay bronchodilator agents used in the treatment of asthma. Recently it has been suggested that some of the variability observed in response to these agents may be due to genetic polymorphisms.[1-4] The β_2-adrenoceptor locus on chromosome 5q31 contains a number of single nucleotide polymorphisms (SNPs). Within the coding region of the human β_2-adrenoceptor gene itself, 9 SNPs have been identified[5], 5 of which are degenerate. Non degenerate polymorphisms result in amino acid substitutions in codon 16(Arg16→Gly), 27(Gln27→Glu), 34(Val34→Met), and 164(Thr164→Ile). In recombinant and non recombinant cell systems the Gly16 variant shows enhanced downregulation whereas the Glu27 variant is partially protected from downregulation[6,7]. The Ile164 variant whilst rare (allelic frequency ~2% in Caucasian populations) reduces the efficiency of receptor coupling with downstream effector pathways.[8] In vivo there are data suggesting a functional role in fibroblast cell lines and cultured human airway smooth muscle for the polymorphisms at codon 16 and 164 and possibly also 27 (reviewed by Hall[9]).

Recent studies have also demonstrated the presence of a number of polymorphisms within the 5′ untranslated region (UTR) of the human β_2-adrenoceptor gene. In a previous study[10] we described 8 SNPs within a 1.5kb region upstream from the ATG start codon This region is believed to be important for regulation of β_2 adrenoceptor gene transcription: it contains the majority of promoter activity for the human β_2-adrenoceptor gene and also includes a short open reading frame (sORF) for a 19 amino acid peptide known either as beta upstream peptide (BUP) or the β_2-adrenoceptor 5′ leader cistron.[11] Using a reporter gene strategy we demonstrated that a construct containing the most frequently occurring non-wild type haplotype for the 4 SNPs contained within the 550bp region of the 5′ UTR showed reduced luciferase expression in COS 7 cells compared to wild-type.[12] This 550bp region contains the majority of promoter activity in the 5′UTR of the β_2-adrenoceptor gene. McGraw et al.have also reported reduced β_2-adrenoceptor expression with the BUP Cys19→Arg polymorphism in a recombinant cell system where the β_2-adrenoceptor was expressed downstream of either the Cys19 or Arg19 form of BUP. More recent work, however, has suggested that when haplotypes (i.e. combination of polymorphisms across this region) rather than single polymorphisms are considered the commonest haplotype containing the Cys19 polymorphism actually is associated with higher levels of β_2-adrenoceptor expression in a recombinant cell system.[13] In contrast, in a preliminary study in primary cultured human airway smooth muscle cells we were unable to detect significant effects of any of four 5′UTR SNPs on expression of firefly luciferase either when each SNP was studied in isolation or in combination using the most frequently occurring haplotypes across this region.[14] Hence, whilst functional data in recombinant cell systems suggest a potential role for the β_2-adrenoceptor 5′UTR polymorphisms, their importance to responses in subjects with or without asthma remains unclear.

In this study we concentrated on haplotypes and the two 5'SNPs most likely to be functionally important. The first of these SNPs is due to a base change 47bp upstream from the β adrenoceptor gene start codon (–47 T-C) which, as discussed above substitutes an Arg for a Cys in BUP. The second 5'UTR SNP which appears potentially to be important is due to base change (T-C) at –367bp from the start codon. This interrupts a putative Sp1 binding site in a region of the promoter containing strong positive promoter activity. To assess the potential relevance of these two 5'UTR polymorphisms and the known common polymorphisms in the coding region of the gene we set out to achieve three aims. First, we wanted to define the allelic frequencies of the 5'UTR polymorphisms in individuals with asthma. Secondly, we wanted to define the extent of linkage disequilibrium between these 5' UTR SNPs and those within the coding region and determine the commonest haplotypes in the Caucasian population. Finally, in order to assess the potential functional effects of 5'UTR polymorphism on β_2-adrenoceptor expression in vivo, we studied levels of β_2-adrenoceptor expression and cAMP responses to β_2-agonist in human peripheral blood mononuclear cells in relation to genotype. Our hypotheses were that individuals carrying the –47 T-C variant either in isolation or as part of an extended pro-downregulatory haplotype, would have lower resting levels of β_2-adrenoceptors on circulating peripheral blood mononuclear cells.

Methods

Subjects

Two populations of asthmatic subjects were used for these studies from Dundee, UK and Groningen, the Netherlands.

Dundee

A total of 58 patients from the Dundee centre were used, with mean (SD) age 35 (14) years, FEV_1 2.58 (0.88) litres, which was 75.6 (17.9) percent predicted. Forty-eight percent of subjects were atopic, all 58 patients were taking inhaled corticosteroid therapy, 32 with beclomethasone dipropionate at a mean dose 522 (456) µg per day, 18 budesonide at a mean dose of 967 (550) µg per day, and 8 with fluticasone propionate at a mean dose of 1500 (623) µg per day. 20 patients were using long-acting β_2-agonists, 11 with salmeterol, 8 with formoterol and 1 with bambuterol. All long-acting β_2-agonist therapy was withdrawn for a washout period of at least 1 week prior to measurement of lymphocytes β_2-adrenoceptor binding and isoproterenol stimulated cyclic AMP responses. Short-acting β_2-agonists were withdrawn for at least 8 hours. No patients had oral steroids for at least 3 months prior to measurements. The study was approved by the trial ethics committee.

Groningen

The Dutch patients were taken from a randomised double blind parallel trial on the treatment of nocturnal asthma.[15] Fifty patients entered the study, and DNA was obtained from 38 individuals.[15] The mean FEV_1 was 3.37 (0.104) litres (86.97 (16.6) percent predicted), and mean PC_{20} (methacholine) 1.78 mg/ml for these individuals. Patients were included if they were non-smoking, atopic asthmatic subjects aged 18 to 45 years, reported a history of episodic dyspnea or wheezing, and showed bronchial hyperresponsiveness to methacholine bromide (PC_{20} < 9.6 mg/ml). Inhaled corticosteroids were stopped 4 weeks prior to study, oral corticosteroids 2 months, and inhaled long-acting β_2-agonists for 2 weeks. All data shown in this paper were taken from the baseline measurement prior to the start of study medication. The genetic part of the study was approved by the Medical Ethics committee of the University Hospital Groningen and additional consent was obtained from all participants.

Isolation of peripheral blood mononuclear cells

30-40mls of blood were collected into tubes containing EDTA and diluted to 50 ml with phosphate buffered saline. Equal aliquots of diluted blood were then layered carefully onto 15 ml of lymphoprep (Nycomed Pharma AS, Oslo, Norway) and centrifuged. A lymphocyte layer was then removed from each tube and combined before 2 further washes with phosphate buffered saline and centrifugation. The supernatant was discarded and the lymphocyte pellet resuspended in 5 mls of phosphate buffered saline or Tris-buffer (Groningen). The preparation was counted to determine lymphocyte numbers and a sufficient volume was removed in order to obtain a total of 2.5 x 10^6 (Dendee) or 10^7 cells (Groningen) cells which was required for cyclic AMP stimulation to isoprenaline. The remainder of the suspension was centrifuged and the supernatant discarded and the lymphocyte pellet resuspended in the suspension buffer to give a concentration of 2.5 x 10^6 (Groningen) or 5 x 10^6 cells per ml.

Genotyping

Polymerase chain reaction (PCR) was used to amplify the β_2-adrenoceptor regions of interest. Table 1 shows the primers and conditions used for each loci. Coding region polymorphisms at codon 16 and 27 were genotyped using allele specific oligonucleotide (ASO) hybridisation as previously described 16. The –47 polymorphism within BUP was genotyped using a PCR based restriction length polymorphism (RFLP) assay. A *Msp*A1 I restriction site is present in the Arg19 (C) form which is not present in the Cys19 (T) sequence. The –367 polymorphism was also genotyped using RFLP, a *Bsu*36 I site is present in the sequence containing the T allele while the C allele obliterates the recognition sequence of *Bsu*36 I.

Table 1 PCR primers and conditions used to amplify the regions of interest for further analysis, the expected fragment lengths are also indicated.

Polymorphism	Primers	Conditions	Fragment Length
Promoter -367	**Forward** **5'** CCTCTGCCTCGAGACCTCAAGCC **3'** **Reverse** **5'** CCGTCTGCAGACGCTCGAAC **3'**	60°C annealing & 30 cycles	740bp
Promoter -47 Arg19 → Cys	**Forward** **5'**CTTCGCGGCTGCCGGCGTG**3'** **Reverse** **5'** GACATGGAAGCGGCCCTCAG **3'**	68°C annealing & 34 cycles	1031bp
Coding Arg16 → Gly Coding Gln27 → Glu	**Forward** **5'**CCCAGCCAGTGCGCTTACCT **3'** **Reverse** **5'** CCGTCTGCAGACGCTCGAAC **3'**	60°C annealing & 36 cycles	234bp

Measurement of cyclic AMP responses and β_2-adrenoceptor binding in peripheral blood mononuclear cells

Dundee methods

Lymphocyte β_2-adrenoceptor binding affinity (Kd) maximum binding density (Bmax) were assayed on the prepared cell suspension (see above) after incubation in a 37°C waterbath in tubes containing (-)^{125} I-iodocyanopindolol (ICYP) at 8 concentrations from 5 to 160pM. Half the tubes contained CGP 12177A HCl (1mM) to prevent ICYP binding to the receptor sites. After washing with assay buffer the bound and unbound preparation of suspensions were aspirated onto filter paper using a Brandel cell harvester and the resultant counts determined by gamma counter. Specific receptor binding was calculated from total binding minus non-specific binding. Receptor density was calculated by Scatchard analysis using the specific and non-specific binding curves plotted for each concentration of ICYP. The intra-assay coefficient of variation for analytic imprecision was 5.8% for Kd and 10.3% for Bmax.

Cyclic AMP was determined for Dundee samples as follows. The suspension containing 5 x 10^6 cells was centrifuged and the pellet resuspended in phosphate buffered saline containing theophylline 100 µM and bovine serum albumin (10%). It was then stimulated with isoprenaline 10^{-4}M during the incubation at 37°C before terminating the reaction by heating to 95°C. After centrifugation the supernatant was removed and cyclic AMP was determined by radioimmunoassay. The intra-assay coefficient of variation for analytical imprecision was 2.0%.

The methods used for samples from Groningen were similar. In brief, samples of 2.5 x 10^6 cells in 900ml Tris buffer containing 0.5 mM 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity were preincubated for

10 minutes at 37°C. After the preincubation step, 100ml isoprenaline (final concentration 1mM) was added to duplicate samples. Basal cAMP levels were determined in duplicate by adding 100ml buffer solution. For cAMP accumulation the samples were incubated for another 10 minutes at 37°C. The reaction was stopped by adding 100 ml 2 M HC, 0.1 M EDTA, followed by heating at 80°C for 10 minutes. After centrifugation, the supernatants were collected and neutralised with $CaCO_3$.[17] After removing excess of $CaCO_3$, cAMP concentration was measured using an immunoassay (Biotrak, Amersham, UK).
Results obtained from each centre were pooled for the analysis by genotype.

Statistical methods

Haplotype frequency estimation across the 4 loci was tested, using the Estimated Haplotypes (EH) program by Ott and colleagues.[18,19] Expected and observed frequencies were compared using log likelihood methods. Possible associations between genotypes/haplotypes and clinical phenotypes were tested, using one-way analysis of variance (ANOVA).

Results

Allelic frequencies of the -367 T/C and –47 T/C β_2-adrenoceptor polymorphisms and linkage disequilibrium

We found that both the -47 T-C and -367 T-C SNPs are common in the Caucasian populations studied. Data showing the allelic frequencies of these polymorphisms are shown in table 2 and observed haplotype data in table 3. It can be seen that the polymorphisms at bp –367 and –bp47 are in strong linkage disequilibrium and that linkage disequilibrium also exists between these polymorphisms and those at codons 16 and 27 within the coding region of the gene (table 4). Allele frequencies were not significantly different in the two populations studied and hence data were pooled for all subsequent analyses.

Table 2 Allele frequencies of the β_2-AR polymorphisms, -367 and -47 in the 5' flanking region, loci 16 and 27 in the coding region (n = 96).

Loci	Allele 1		Allele 2	
-367 T/C	T	0.62	C	0.38
-47 T/C	T	0.60	C	0.40
Arg16 → Gly	Arg16	0.31	Gly16	0.69
Gln27 → Glu	Gln27	0.48	Gln27	0.52

Table 3 Individual haplotypes observed in the asthmatic population for which all loci were genotyped (n = 93).

-367 (T/C)	-47 (T/C)	Arg16 → Gly	Gln27 → Glu	No.	% Total
C/C	C/C (Arg19)	Gly16	Glu27	19	20
T/T	T/T (Cys19)	Het	Gln27	10	11
T/T	T/T (Cys19)	Arg16	Gln27	9	10
T/C	T/C (Het)	Het	Het	8	9
T/T	T/T (Cys19)	Het	Het	8	9
T/C	T/C (Het)	Gly16	Het	7	7
T/T	T/C (Het)	Gly16	Het	4	4
T/T	T/T (Cys19)	Gly16	Glu27	3	3
T/T	T/T (Cys19)	Gly16	Het	3	3
T/C	T/C (Het)	Gly16	Glu27	3	3
C/C	C/C (Arg19)	Het	Het	3	3
T/T	T/C (Het)	Het	Het	3	3
T/C	T/T (Cys19)	Gly16	Glu27	2	2
T/C	T/C (Het)	Het	Gln27	2	2
T/T	T/T (Cys19)	Gly16	Gln27	2	2
T/C	T/T (Cys19)	Het	Het	2	2
T/T	T/T (Cys19)	Arg16	Het	1	1
T/T	C/C (Arg19)	Het	Gln27	1	1
T/C	C/C (Arg19)	Gly16	Het	1	1
T/T	T/C (Het)	Gly16	Glu27	1	1
T/C	T/T (Cys19)	Gly16	Gln27	1	1

Table 4 Haplotype frequencies in β_2-adrenoceptor gene

HAPLOTYPE				FREQUENCY	
-367 (T/C)	-47 (C/T)	Arg16 → Gly	Gln27 → Glu	With association	Without association
C	C (Arg19)	Gly16	Glu27	0.32	0.06
T	T (Cys19)	Arg16	Gln27	0.26	0.05
T	T (Cys19)	Gly16	Gln27	0.16	0.12
T	T (Cys19)	Gly16	Glu27	0.13	0.13
T	C (Arg19)	Gly16	Glu27	0.036	0.091
C	C (Arg19)	Arg16	Gln27	0.029	0.023
C	T (Cys19)	Gly16	Glu27	0.022	0.079
T	C (Arg19)	Gly16	Gln27	0.017	0.089
T	T (Cys19)	Arg16	Glu27	0.007	0.058
T	C (Arg19)	Arg16	Gln27	0.007	0.038
C	T (Cys19)	Gly16	Gln27	0.007	0.075
C	C (Arg19)	Gly16	Gln27	0.00006	0.052

Haplotype frequencies determined from the data in table 3 using the Estimated Haplotype program to estimate the haplotype combinations of the double, triple and quadruple heterozygous genotypes, both with and without association. The Ln (L) χ^2 statistic 249.08 d.f 11 (p = 0.00008) to suggest strong linkage disequilibrium across these loci. Four haplotypes did not occur with association.

Table 5 β_2-adrenoceptor levels of receptor expression (B max), receptor affinity (KD), and the ability of isoprenaline to stimulate cyclic AMP formation by β_2-adrenoceptor genotype

Polymorphism		Averages					
Loci	**Genotype**	**Bmax fmol/mg**	**ANOVA P values**	**KD pmol**	**ANOVA P values**	**Maximum response to Isoprenaline Emax (% above basal)**	**ANOVA P values**
-367 T/C	T/T	2.75 ± 0.32	0.27	21.5 ± 2.26	0.71	289 ± 27.69	0.01*
	T/C	2.17 ± 0.17		19.5 ± 1.80		452 ± 57.27	
	C/C	2.43 ± 0.22		21.3 ± 1.69		327 ± 39.30	
-47 T/C	T/T	2.78 ± 0.32	0.27	20.5 ± 2.36	0.98	305 ± 28.37	0.17
	C/T	2.38 ± 0.19		20.9 ± 1.89		409 ± 57.78	
	C/C	2.27 ± 0.18		20.4 ± 1.54		331 ± 37.55	
Arg16 → Gly	Arg16	2.55 ± 0.22	0.73	25.2 ± 3.24	0.11	290 ± 71.58	0.68
	HET	2.54 ± 0.17		21.3 ± 1.40		363 ± 40.23	
	Gly16	2.33 ± 0.24		18.6 ± 1.65		339 ± 31.48	
Gln27 → Glu	Gln27	2.48 ± 0.23	0.26	22.4 ± 1.96	0.54	329 ± 31.26	0.89
	HET	2.22 ± 0.17		19.5 ± 1.73		353 ± 31.89	
	Glu27	2.73 ± 0.29		20.5 ± 1.88		352 ± 30.95	

Mean and Standard Error, β_2-adrenoceptor levels of receptor expression (B max), receptor affinity (KD), and the ability of isoprenaline to stimulate cyclic AMP formation was averaged by genotype groups to test for a particular genotype association to a clinical phenotype (ANOVA). Data was derived from a Dundee population (n = 58) and a Dutch population (n=38) * $p<0.05$.

Effect of β_2-adrenoceptor promoter-coding region haplotype on β_2-adrenoceptor expression and coupling in circulating peripheral blood mononuclear cells

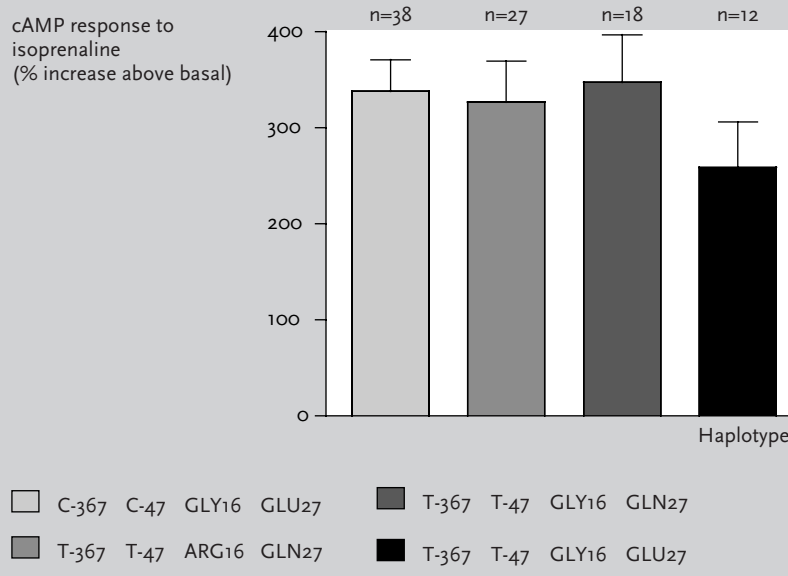
In order to attempt to define potential functional effects of β_2-adrenoceptor polymorphism within the promoter region, we hypothesised that individuals with the Cys19→Arg polymorphism may show reduced levels of β_2-adrenoceptor expression in vivo. We therefore looked at levels of β_2-adrenoceptor expression, affinity and function in peripheral blood mononuclear cells obtained from 58 Scottish asthmatics and 38 Dutch asthmatics (table 5). These data show that when either the BUP Cys19→Arg or the -367 T-C SNP are considered in isolation no clear functional effects of these polymorphisms are evident. As would be predicted, no effect of the Arg16→Gly and Gln27→Glu β_2-adrenoceptor polymorphisms is apparent when considered in isolation (table 5).

Although levels of receptor expression measured by binding provide valuable information, it remains possible that receptor coupling may be altered without an accompanying change in the overall level of receptor expression. Therefore, we examined the ability of PBMCs derived from individuals with different genotypes to generate cyclic AMP in response to isoprenaline. No clear differences in the ability of cells expressing different forms of the β_2-adrenoceptor to generate cAMP response was evident between genotypes (table 5). We did observe a small, statistically significant increase in cyclic AMP responsiveness in those individuals heterozygous for the –367T-C SNP. However, no trend was observed in homozygous individuals to support a role for this SNP and we believe this to represent a false positive result. In a posthoc sub-analysis we also compared the cyclic AMP responsiveness in those receiving inhaled steroid at the time of study (i.e. the 58 Dundee subjects) and those in which inhaled steroid therapy had been discontinued (i.e. the 38 Dutch individuals). Again, no significant differences were observed between groups defined by genotype (data not shown).

Haplotype analysis of β_2-adrenoceptor expression in peripheral blood mononuclear cells

Given our in vitro data suggesting that effects of β_2-adrenoceptor promoter polymorphisms may only be apparent when relevant haplotypes are studied, we attempted to perform a haplotype analysis looking at levels of β_2-adrenoceptor expression and coupling in peripheral blood mononuclear cells studied ex vivo. To maximise the chance of observing effects, we only considered individuals homozygous for all the SNPs or those heterozygous at only a single position. This analysis was inevitably complicated by the strong linkage disequilibrium between these SNPs described above: no individuals with the potentially informative BUP Arg19, β_2-adrenoceptor Gly 16 Gln 27 homozygous haplotype were identified within our population (table 3). No significant difference was observed between levels of expression or cyclic AMP responses to isoprenaline when considered by haplotype (Figure 1), although the numbers are inevitably small for this analysis.

Figure 1 Graph to show % change in cAMP formation in response to isoprenaline stimulation of PBMCs with respect to haplotype.



Discussion

In this paper we describe a structured approach to look for functional effects in vivo of recently described β_2-adrenoceptor promoter polymorphisms using peripheral blood mononuclear cells from patients with asthma. The main conclusions of the study are that:

(i) The 5' UTR β_2-adrenoceptor promoter polymorphisms at –367 (T-C) and –47 (T-C) are common in the Caucasian population.
(ii) Strong linkage disequilibrium exists across this region which results in several common haplotypes occurring.
(iii) No clear individual effect of β_2-adrenoceptor promoter polymorphisms can be seen on the level of expression or coupling of β_2-adrenoceptors in peripheral blood mononuclear cells isolated from patients with asthma.
(iv) No clear functional effect of any given haplotype on the level of receptor expression and coupling in PBMCs could be identified.

The promoter region for the human β_2-adrenoceptor gene (cf. other species such as the rat) has been relatively poorly studied. However, it is clear that promoter activity resides within a region of approximately 1.5kb and that the majority of promoter activity is found in the first 550 base pairs up-

stream of the start codon.[12] This region contains a sORF for BUP which is believed to act as a translational (and possibly transcriptional) inhibition system.[20] Part of the mechanism whereby leader peptides such as BUP exert their effect is believed to depend upon the arginine content of these peptides: the Cys19→Arg polymorphism due to the SNP at –47 (T-C) increases the arginine content of the peptide from 3 to 4 residues out of the total of 19. This increased number of arginine residues would be predicted to increase its ability to exert translational inhibition. Data to support this suggestion have been published previously.[11] However, preliminary data reported by our group suggest that, at least with luciferase expression as an end-point, in primary cultured airway cells the presence of the Cys19→Arg BUP polymorphism is inadequate by itself to cause functional effects.[14] Given these conflicting data, the probable effects in vivo of these SNPs are unclear, and are further complicated by the strong linkage disequilibrium, which exists across this region. Four SNPs have been described in the 550bp region immediately upstream from the ATG which contains the majority of promoter activity. However, the polymorphisms at –468 (C-G) and –20 (T-C) seem unlikely to contribute to functional effects, given that they do not alter known transcription factor binding sites. Therefore, we limited our analysis to the polymorphisms at -367 which is close to an SP1 site, -47 (Cys19→Arg in BUP) and the two common coding region polymorphisms at codon 16 and 27 of the β_2-adrenoceptor gene itself.

Next, we attempted to determine whether β_2-adrenoceptor expression ex vivo is determined by β_2-adrenoceptor promoter polymorphisms. The known functional effects of the codon 16 and 27 β_2-adrenoceptor polymorphisms are to alter downregulation profiles both in recombinant cell systems 6 and in primary cultured human airway smooth muscle 7. However, given the in vitro data, we would predict that the –367 (T-C), Cys19→Arg(BUP), Arg16→Gly, Gln27→Glu β_2-adrenoceptor promoter coding region haplotype might lead to reduced expression of β_2-adrenoceptors in vivo without agonist exposure which might increase following agonist exposure: this would also be predicted from McGraw et al's data. We attempted to study this by looking at levels of β_2-adrenoceptor expression on circulating peripheral blood mononuclear cells isolated from patients with asthma. However, when considered in isolation, none of the above polymorphisms altered levels of receptor expression (B max) or receptor affinity (KD). In addition, no difference in the ability of isoprenaline to drive cyclic AMP formation in these cells was observed when different genotypes were considered in isolation. We did observe a small difference in individuals heterozygous for the –367T-C SNP but given that no effect was seen in the two relevant homozygous groups this seems likely to be a false positive result. In a secondary analysis we also looked to see if there was evidence that inhaled steroid therapy (used in the Dundee subjects) may have masked any effect of genotype on cyclic AMP responsiveness. However, when analysed by subjects defined by inhaled steroid therapy, no

significant effects were apparent, although the power of this analysis is obviously smaller than for the main study. Similarly, down regulation was not more apparent in subsets of the nocturnal asthma group defined by genotype or haplotype. Previously, we have shown that inhaled steroid therapy does not protect against β_2-adrenoceptor bronchodilator desensitisation [21], although effects have been observed on β_2-adrenoceptor mediated protection against bronchoconstriction.[22]

One explanation for the lack of effect when each SNP is analysed in isolation is that functional differences are only present in individuals with a particular haplotype across this region. We were able to demonstrate strong linkage disequilibrium across this region, with the most frequently occurring haplotypes being –367 C, Arg19BUP, Gly16, Glu27 and –367 T, Cys19 BUP, Arg16, Gln27. We therefore examined the effects of different β_2-adrenoceptor haplotypes covering both the promoter and the functio nal polymorphisms within the coding region on β_2-adrenoceptor expression and coupling in PBMCs. Inevitably, this analysis was complicated by the lack of individuals with some potentially informative haplotypes: however, our data suggest that there is unlikely to be a marked effect of β_2-adrenoceptor promoter polymorphism upon β_2-adrenoceptor expression at least in peripheral blood mononuclear cells. It is of note that 42% of all the individuals studied, where haplotype could be determined, carried one of 2 major haplotypes across this region. Hence, even if a given (rarer) haplotype were important in determining the level of expression and coupling of the β_2-adrenoceptor in PBMCs, very large population samples, or samples from ethnic groups in which the SNP frequencies are different, would be required to fully address this issue. Whilst preparing this manuscript a further study addressing this issue was published demonstrating strong lin kage disequilibrium across this region in a USA population.[13] This study also suggested that haplotypes rather than individual SNPs might predict bronchodilator reversibility, although the numbers were small.

In order to obtain the number of cells from a reasonable number of individuals necessary for this study we elected to use peripheral blood mononuclear cells as our assay system. Some studies have suggested that these may not be the best surrogate for studying β_2-adrenoceptor expression in the lung. However, desensitization and/or downregulation has generally been easier to demonstrate in circulating PBMC than in airway cells, and hence it would seem unlikely that marked genotype/haplotype dependent effects would be seen in airway cells but not in PBMCs. It would be difficult to obtain pulmonary cells from adequate numbers of individuals with informative haplotypes to repeat this study using, for example, bronchoepithelial cell β_2-adrenoceptor expression as an appropriate endpoint.

A preliminary assessment of the potential contribution of β_2-adrenoceptor promoter/coding region haplotypes to treatment response (defined as ΔFEV_1 to Salbutamol) was able to show differences between groups defined by haplotypes, although the groups with the worst responses and best responses were not those that one would predict from in vitro functional studies.[13] In a post-hoc analysis we examined the degree of baseline reversibility in the subjects from Groningen for whom reversibility data and DNA were available (n = 30, mean percent reversibility 16.0%). No significant associations were seen when genotypes were analysed individually (n = 30) or as haplotypes (n = 13).

One possible criticism of this study is the use of two different patient po pulations. However, asthma severity was comparable between the groups gauged by FEV_1 and previous medication. No significant differences in genotype distribution were evident between the populations in the two centres; the genotype frequencies being in keeping with previous data on β_2 adrenoceptor polymorphism frequencies in other caucasian populations. Therefore we believe it unlikely that our results are influenced by the pooling of data obtained in Dundee and Groningen.

In summary, we have shown that β_2-adrenoceptor promoter polymorphisms are common in the Caucasian population. Strong linkage disequilibrium exists between these promoter polymorphisms (and those within the coding region of the β_2-adrenoceptor) resulting in the occurrence of several common haplotypes. However, using PBMC β_2-adrenoceptor expression and coupling as functional end points, we were unable to demonstrate marked functional consequences in vivo, of any individual SNP or of the commonest combinations studied using a haplotype approach. It is unlikely therefore, that β_2 promoter polymorphisms play a major role in determining basal levels of β_2-adrenoceptor expression and coupling in vivo.

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Summary and future perspectives

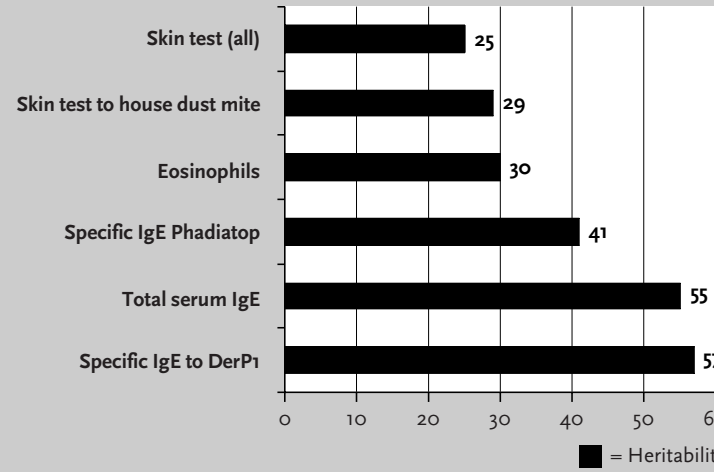
Asthma and atopy are prevalent chronic diseases that affect millions of people worldwide, and these prevalence rates are increasing all over the world. Thus, it is important to unravel the pathophysiological determinants of their development. Asthma and atopy are caused by an interaction of genes and environmental factors. This thesis deals with host factors; that is the genetics of asthma and atopy.
This thesis comprises studies performed by a multidisciplinary collaboration between universities in the Netherlands (University of Groningen) and the United States of America (Wake Forest University, Winston-Salem and the University of Maryland at Baltimore) to unravel the complex nature of asthma and atopy. In this chapter the main conclusions of this thesis will be summarized. Every part of this thesis describes a step in genetic research, from the disease and its definition (part 1), the determination of the genetic and environmental contribution (part 2), the identification of chromosomal regions that may contain asthma and atopy genes (part 3), the study of candidate genes (part 4) to the investigation of gene function (part 5). For every part, recommendations for future research are given.

In the first part a review of current published evidence on the genetics of asthma and atopy is presented. From this review, it is clear that progress in genetics has been made in the years prior to the start of this study. First, the genetic contribution to asthma and atopy has been shown repeatedly with twin and family studies. It is clear that this genetic contribution consists of multiple genes, interacting with each other and with the environment. Second, chromosomal regions on human chromosomes 5, 11, and 12 had been identified at that time, which are most likely to contain asthma and/or atopy genes. Third, candidate gene analyses had shown genetic associations of alleles in the genes encoding the FCεRIβ, cytokine genes (IL-4, IL-9, IL-13) and their receptors (e.g. IL4 receptor gene) in asthma and / or atopy. Replication of linkage as well as association results has not been proven easy so far. Differences in ascertainment strategies, types of populations, sample sizes, and phenotype definitions may explain this difficulty. Finally, functional data showing a change in quantity or quality of the gene product in relationship to a proposed single nucleotide polymorphism in a candidate gene is present for only a few genes.
An important first step in genetic research is the definition of the disease. Since a gold standard for diagnosing asthma is lacking, the best possible approach is to diagnose asthma with the use of validated questionnaires in combination with objective markers of variable airway obstruction, bronchial hyperresponsiveness and / or airway inflammation. In addition, the recommendations of Lander and Kruglyak[1] for defining diseases in genetic studies may be noteworthy. These authors advise one to study a subset of the disease such as early age of onset, which has been very useful in cancer genetics; to define a severe disease phenotype; (if possible) to choose a specific sub-phenotype, and choose patients with a positive fami-

ly history.[1] Application of these four recommendations to narrow the disease definition would lead to the selection of the following category of asthma patients for genetic studies: asthma with early age of onset, ongoing into adulthood, severe asthma, in individuals with a positive family history. However, evidence for familial aggregation of a specific subphenotype of asthma, such as early age at onset, has not been provided to date. A very interesting phenotype is the 'longitudinal' phenotype, i.e. the outcome of asthma. For instance, it is not fully understood what predicts the progressive loss of lung function in asthma. Some risk factors for development of low lung function have been identified, such as low initial FEV_1, bronchial hyperresponsiveness and smoking.[2,3] However, the genetic contribution to the outcome of asthma is unknown. We recommend to study which genes have a modifying effect on the outcome of asthma. Interesting candidate genes could be IL-4, IL-4 receptor, and IL-13. For IL-4 and IL-4 receptor, cross sectional data are published showing associations of alleles in these genes with low lung function.[4,5] For IL-13, a transgenic mouse model overexpressing IL-13 showed signs of inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production.[6] If this would apply to the human situation, one could predict that individuals with asthma expressing larger quantities of IL-13 (i.e. individuals homozygous for the –1111 T allele),[7] would have a higher risk of developing low lung function and irreversible airway obstruction in later life.

The second part of this thesis focuses on the determination of the genetic contribution to asthma and atopy. Multiple twin studies, such as the study presented by Skadhauge et al.[8], have indicated that the genetic contribution to asthma is considerable. Twin studies that use genetic modeling indicate that the genetic variability in asthma is composed of additive genetic factors, in combination with a polygenic component. In our family study, a clear familial clustering of atopic traits (total serum IgE, specific IgE to aeroallergens, skin test positivity and blood eosinophil levels) was shown. The heritability of these traits was estimated using variance components analysis (figure 1). Heritability estimates (h^2) were highest for specific IgE to Der P1 (0.57) and for total serum IgE (0.55); h^2 was 0.41 for Phadiatop; 0.30 for log eosinophil count; 0.29 for skin test to house dust mite, and 0.25 for skin test positivity.
Twin studies that use genetic modeling provide evidence for the type of environmental factors that may be important. Two types of environmental factors are distinguished: individual specific factors that interact specifically with an individual, and shared factors that act for both members of a twin pair. According to twin studies in asthma, the environmental contribution to asthma appears to be mainly individual specific, but not shared. Thus, in asthma genetic factors may interact with individual specific environmental factors in a unique fashion, i.e. dose and / or time dependent. Now that candidate genes for asthma and atopy are becoming clearer, large-scale studies of gene by environmental interaction should be

Figure 1. Heritability of asthma- and atopy associated traits in the Dutch family study as calculated by variance components analysis.



planned. Important environmental factors to be mentioned are active and passive smoking, allergen and endotoxin exposure. Another interesting environmental factor is indicated by the sibling effect, which is reported in the second part of the thesis. In our family study, the presence and severity of atopy was inversely associated with the size of the family (specific IgE to common aeroallergens) and birth order (skin test positivity). It is currently believed that the sibling effect is a proxy of the presence of childhood infections. For future intervention studies, it is important to realize that even in these high-risk families, environmental effects may modify atopy.

We believe that a better understanding of asthma and atopy will be provided by studies of gene by environmental interaction. In our family study, we have therefore started to investigate the environment by collection of house dust for analysis of house dust mite allergens and lipopolysacherides. We recommend performing studies on the interaction of allergen exposure with the specific immune response (HLA region), as well as with genes important in the upregulation of the immune response, such as the cytokine genes and their receptors. Finally, interaction of endotoxin with its receptors, such as CD14, could be studied in relationship to the development or modification of the allergic response. In addition, the interaction of endotoxin with the Toll like receptor 4 with respect to asthma severity and lung function could provide important explanations on the mechanism of endotoxin induced bronchoconstriction and airway inflammation.[9]

The third part of this thesis comprises genome wide linkage results in 200 families ascertained through a proband with asthma. In the past five years, we have added 108 families and restudied 66 of the previously ascertained families in Beatrixoord in Haren, the Netherlands.[10] In the linkage analysis of the total set of 200 families, several chromosomal regions showed evidence for linkage of an atopic phenotype: chromosome 2q, 6p, 7q, and 13q. These also showed evidence of linkage with total serum IgE. Specific regions of interest for atopic traits were also detected at chromosome 11q, 17q, and 22q. Although there was confirmation of chromosomal regions thought to be important in allergy and asthma (e.g. 5q, 12q), also novel regions were detected. The most significant finding was for total serum IgE levels on chromosome 7q, a finding that needs to be followed up in further replication and fine mapping studies, which will be carried out in our department. Another interesting chromosome is chromosome 2q, which contains strong candidate genes for atopy and asthma. We have fine-mapped this region on chromosome 2q. The LOD score for total serum IgE in this region increased from 1.96 to 3.16 with the addition of new markers. Two candidate genes in this region, CTLA-4[11] and CD28, were studied. Significant evidence for association of asthma and atopy was observed with two SNPs in the CTLA-4 gene (table 1).

Table 1. Candidate gene results from this thesis

Gene	Chromosome	SNP	Phenotype	Function
CTLA-4	2q33	-1147 C-T	Asthma, BHR	Possibly influences gene transcription
		-318 C-T	No associations identified	
		+49 A-G	Total serum IgE, asthma, BHR, skin tests	Signal transduction in co-stimulation of T cell activation
CD28	2q33	-824 A-G	No associations identified	
CD14	5q31	- 159 C-T	Total serum IgE and number of skin tests in skin test positive subjects	Possibly upregulates gene transcription
IL 13	5q31	-1111 3'UTR	BHR, asthma, skin test positivity	Possibly upregulates gene transcription
IL4 receptor	16p12	E375A C406R S478P	Total serum IgE, skin test positivity, asthma, BHR	Enhances signal transduction of the receptor

BHR Bronchial hyperresponsiveness

The next challenge is to identify genes that underlie these linkage signals. Experience from the past decade shows that this is a most difficult task in genetic research of complex diseases. In asthma, no studies of successful fine mapping studies have appeared in the literature, but this may change since press releases have announced that some 'asthma genes' have been identified.[12] We have been involved in a collaborative study on positional cloning of a susceptibility gene for bronchial hyperresponsiveness on chromosome 5q together with Novartis Pharmaceuticals, Horsham, United Kingdom (dr. P. Whittaker) and new findings will be published in the future.

Progress in genetic research may further be enhanced by developments in statistical approaches, novel insights into population characteristics and physical data from the Human Genome Project.

For fine mapping and positional cloning, no gold standard exists with regard to the statistical methodology. The classical approach from Mendelian diseases, which studied families, and identified critical recombinants to narrow down the region can not be used in the genetics of complex diseases. Therefore, the possibility of large-scale association analysis has been discussed in the literature.[13,14] Another interesting method is the analysis of shared haplotypes between cases and controls. In founder populations derived from a limited number common ancestor, it is expected that patients have similar polymorphisms in a disease gene and surrounding ha plotypes than unaffected controls. Several investigators, including Dr. Te Meerman at the University of Groningen are developing statistical me thods that use this principle.[15-18] In the past years, we have collected approximately 250 trio's (patient and two family members) from the province of Friesland to use this methodology. It is therefore important to know the type of population under study (e.g. inbred, founder, or outbred population) and know physical characteristics, such as the level of linkage disequilbrium. From preliminary studies it appears that the Dutch and Friesian population residing in the northern part of the Netherlands have appealing characteristics for genetic research.

Finally, physical data from the Human Genome Project have become available for genetic researchers in 2001. One surprising finding was that the number of genes in the human genome is lower that expected, approximately 30.000. This may sound appealing for gene 'hunters', because a lower gene density may result in a lower number of positional candidate genes, which could make fine mapping easier. However, it has become increasingly likely that normal physiological and possibly pathophysiological processes do not depend on the function of a single gene, but that multiple genes may interact. Thus, it may be anticipated that complex gene-gene interaction studies need to be carried in the future. It may be predicted that studies of function of the transcriptome and proteome will be important next steps in understanding these interactions and providing models for intervention.

In part four of this thesis, three candidate genes are analyzed (table 1). First, evidence is presented for a –159 C to T promoter polymorphism in the CD14 gene (CD14/-159) modifying the severity of allergy, as expressed by higher serum total IgE levels and higher number of skin tests in skin test positive individuals. This confirmed data presented by Baldini et al. from the Tucson study in the United States. In addition, we showed that individuals carrying two C alleles at CD14/-159 were more likely to develop hayfever and allergic rhinitis, but not asthma.
Second, we investigated IL-13 as a candidate gene for asthma. We analyzed three single nucleotide polymorphisms (SNPs) in IL13 in an extended group of 184 probands and spouses: one in the promoter region (-1111), the Arg130Gln (nucleotide position 4257), and a 3' UTR SNP (nucleotide position 4738). The most significant associations were observed to asthma (p=0.005), bronchial hyperresponsiveness (p=0.003), and skin-test responsiveness (p=0.03) with the –1111 promoter, replicating previous associations with asthma and allergy phenotypes.[19,20,21] These results provide evidence that variation in the IL13 gene is involved in the pathogenesis of asthma and atopy. Further replications and functional analyses are needed to clarify the possible role of coding and regulatory SNPs in this gene.
Third, an investigation of the different SNPs in the interleukin 4-receptor gene is presented. Significant associations of three tightly linked SNPs in exon 12 of this gene with total serum IgE and skin test positivity was identified. This further confirms published data of Ober et al.[22] and Kruse et al.[23] indicating a role of these SNPs acting alone or in concert in the development of atopy. An important finding was that individuals carrying the major susceptibility alleles in IL-4 R (for IgE) and IL-13 (for bronchial hyperresponsiveness) were 5 times more likely to develop asthma than individuals not carrying the risk alleles. This is to our knowledge the first finding of gene-gene interaction in the development of asthma. Since the percentage of individuals carrying both risk alleles in our population was rather small, replication of this finding in other studies is needed.

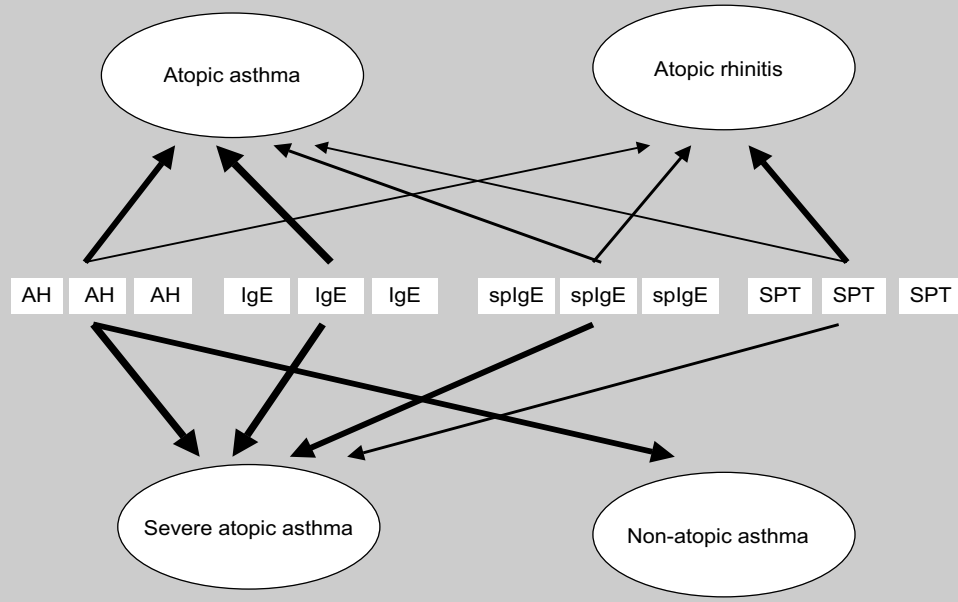
Part five of this thesis comprises functional analysis of the β_2-adrenoceptor. Data showing that the presence of a gene variant alters the function of a gene product in a relevant study system is scanty in the literature. For the β_2-adrenoceptor, a collaborative study was performed together with the Universities of Nottingham (Prof. I.P. Hall) and the University of Aberdeen (Prof. B. Lipworth), United Kingdom. Within the gene encoding the β_2-adrenoceptor, extensive linkage disequiblibrium between coding and promoter variants was shown in different European populations. However, no differences were seen with respect to β_2-adrenoceptor density on circulating peripheral blood mononuclear cells and production of cAMP by peripheral blood mononuclear cells of asthmatics.

Future perspectives

What will genetics bring with regard to understanding the complex pathogenesis of asthma and atopy, the diagnosis and therapy of patients with an atopic disease?

From results of genetic studies, novel insights may arise to understand the complex interrelation between asthma and atopy. From twin and family studies it is suggested that asthma and atopy may have a common genetic basis. In addition, a clustering of linkage signals can be observed for both asthmatic and atopic phenotypes, which may indicate the presence of genes for both phenotypes.[24] In addition, also disease specific genes may contribute to asthma or other atopic diseases. This is suggested from several observations. First, there is no complete overlap of the presence of different atopic phenotypes within families. Second, genome-wide linkage studies also indicate specific regions that show some evidence of linkage to specific atopic phenotypes. Third, different intermediate phenotypes show different associations with asthma (total serum IgE) and allergic rhinitis (skin test positivity). Thus, also specific genes may contribute to different atopic phenotypes and eventually lead to different atopic diseases (figure 2).

Figure 2 Possible interactions between genes for atopy in asthma and rhinitis.



AH Airway hyperresponsiveness; IgE total serum IgE; spIgE specific IgE; SPT skin prick test.
From: D.S. Postma, G.H. Koppelman and D.A. Meyers: The genetics of atopy and airway hyperresponsiveness. Am J Resp Crit Care Medicine 2000; 162: S118-S123.
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To further understand genetic similarities and differences between susceptibility to asthma and atopy, two different strategies can be pursued. First, non-atopic asthma can be studied, which was shown to have a genetic contribution.[25] However, given the fact that only a minority of patients with asthma are non-atopic, this probably needs to be a multi-center study. Second, atopic non-asthmatic patients (for example with allergic rhinitis) may be studied and the genetic results compared to atopic asthmatic patients. Currently, the latter study is being carried out in our department.

An important question is whether genetics will improve (early) diagnosis of asthma? In the majority of children under the age of 6, no lung function measurements or assessments of bronchial hyperresponsiveness can be performed. Therefore, the diagnosis of young children with recurrent episodes of wheezing as having asthma is difficult. Could genetics bring a solution to this problem in the end? It is likely that susceptibility genes for asthma have a high population frequency, given the high prevalence of asthma. If the frequency of this risk allele is for example 30 %, it can be a major contributor to the disease at a population level. If carriers of this risk allele have a relative risk (RR) of 2 to become asthmatic, this genotype alone could explain 23.1 % of the disease on a population level. The question remains if the detection of this allele also gives valuable diagnostic information on a patient level. In the same example (allele frequency 30% and a relative risk of 2), the chance that an individual will develop asthma is only 7.7 % when carrying the risk genotype.[26] Thus, the diagnostic value will be low for an individual patient when assessing genotypes with high population frequencies and low relative risks.[26] However, combination of alleles in different genes (as indicated in our study for IL13 and IL4R) may be more informative.

Will genetics improve management of patients with asthma? Currently, three lines of treatments are available for patients with asthma: inhaled glucocorticoids; bronchodilators and leukotriene inhibitors. Since the res ponse to these drugs is markedly variable between individuals, researchers are now exploring the genetic contribution to the response, but in particular, to non-response to treatments.[27] If indeed, variations in drug targets are of major importance in drug response, then identification of the variants before start of treatment may prevent overtreatment in individuals prone to non-responding. Second, it is currently unclear if genetic factors are important in patients with steroid–resistant asthma. This is an important group of patients with difficult-to-treat, mostly severe asthma.[28] Steroid resistant asthma imposes a heavy burden on the individual patients in terms of morbidity and hospitalizations and thus contributes significantly to the costs of asthma for the society. Unraveling (genetic) factors in steroid resistance could therefore be important in designing new adjuvant strategies in the treatment of these patients. Finally, novel therapeutic approaches with monoclonal antibodies and soluble receptors are now being undertaken.

Especially the experiments with soluble IL4R as a treatment for asthma have attracted our attention.[29] In our genetic studies, a subpopulation carrying the risk genotypes in IL4R and IL-13 genes appear to be at especially high risk for developing asthma. We propose to perform a pharmacogenetic study with soluble IL4R in patients with these risk genotypes. One may predict that blocking IL13-IL4R pathway in this subgroup may be very beneficial. We hope that this may be an illustration of how genetic research could benefit individual patients with asthma.

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Samenvatting in het Nederlands
De genetica van astma en allergie

(Naar Koppelman GH en Postma DS. De genetica van atopie. Ned Tijdschr Allergie 2001;1:5-13)

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In deze samenvatting wordt een overzicht gegeven van de stand van zaken in het onderzoek naar erfelijke oorzaken van astma en allergie. Na definiëring van begrippen die in deze samenvatting gebruikt worden, komt de beschikbare kennis over de chromosomale ligging van genen voor allergie en een aantal kandidaat-genen aan de orde. Steeds zal worden aangegeven wat de onderzoeken die beschreven zijn in dit proefschrift hebben bijgedragen aan deze kennis. We eindigen met een bespreking van mogelijke toepassingen van genetisch onderzoek in de dagelijkse praktijk.

Astma en allergie

Mensen met astma hebben last van benauwdheid, een piepende ademhaling, hoesten en het opgeven van slijm. Dit komt vaak 's nachts voor. Astma wordt veroorzaakt door een ontsteking van de luchtwegen, die leidt tot overgevoeligheid van deze luchtwegen voor prikkels van buitenaf, zoals koude, rook en mist. Bij inademing van deze prikkels vernauwen de luchtwegen zich; men ervaart dan benauwdheid. Astma en allergie gaan vaak samen. Bij allergie reageert het lichaam op kleine stukjes eiwit uit de buitenwereld (allergenen). Hierbij gaat het bijvoorbeeld om huisstofmijt, gras- of boompollen, kat of hond. Na inhalatie van de allergenen maakt het lichaam antistoffen, die imuunglobuline E (IgE) genoemd worden. Bij mensen met allergie is dit aan te tonen door IgE in het bloed te meten. Hierbij meten we de totale hoeveelheid IgE in het bloed of specifiek IgE tegen een bepaald allergeen, zoals bijvoorbeeld de huisstofmijt. Ook meten we allergie door middel van huidtesten. Hierbij wordt een klein beetje allergeen in de huid gespoten. Bij allergie wordt de huid daarna rood en dik. Ook komt bij allergie en astma een bepaald soort witte bloedcellen (de eosinofiele granulocyt) verhoogd voor vergeleken met niet-allergische mensen. Deze eosinofiele granulocyten spelen waarschijnlijk een belangrijke rol speelt bij allergische ontstekingen. Allergie kan tot uiting komen in de longen (astma), in de neus en ogen (hooikoorts) en in de huid (eczeem). Dit proefschrift richt zich voornamelijk op de genetische achtergrond van astma en allergie.
Waarom is het belangrijk dit onderzoek te doen? Het is belangrijk meer te begrijpen van de oorzaken van astma en allergie, omdat allergische ziekten de laatste jaren wereldwijd toenemen. Voor miljoenen mensen vormen allergische ziekten een belangrijke belemmering in het dagelijks leven. Het is niet bekend waarom allergie zo toeneemt, en meer informatie over het ontstaan van allergie zou kunnen helpen bij het voorkómen van allergie of het ontwikkelen van nieuwe medicijnen.

Erfelijkheid en omgeving bij astma en allergie

In de geschiedenis van de geneeskunde werd al vroeg geconstateerd dat allergie en astma niet willekeurig in de bevolking voorkomen, maar dat het vaak 'in de familie zit'. Zo merkte Sennertus in 1650 op dat zijn vrouw, drie van haar broers en zusters en haar nicht allen leden aan astma (zie Wiener et al.).[1] In 1868 vond Salter bij gemiddeld twee van de vijf patiënten met astma een familielid met astma en hij concludeerde dat dit meer is dan op basis van toeval verwacht mag worden.[2] In 1916 publiceerden Cooke en Vanderveer een eerste grote studie. Zij vergeleken de families van 621 patiënten met enige vorm van allergie met die van een controlegroep van 68 personen. In de groep van 621 patiënten had 48,8 % een familielid met allergie, vergeleken met 14,5 % in de controle groep. Deze auteurs concludeerden dat erfelijkheid bijdraagt aan de ontwikkeling van allergie.[3]

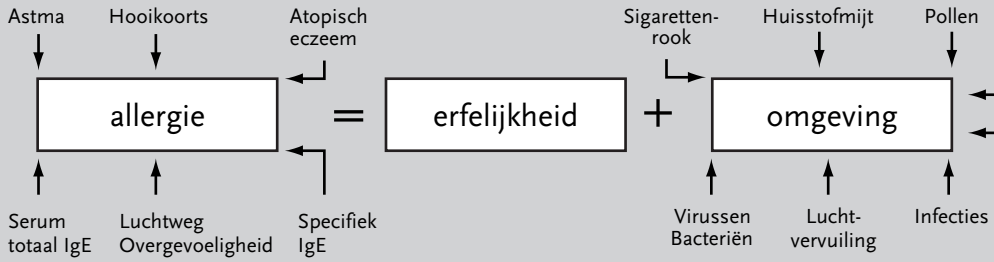
Tabel 1. Allergische ziekte bij een kind in relatie tot een allergische ziekte bij een van de ouders.

Allergische ziekte bij het kind	Astma bij een van de ouders	Hooikoorts bij een van de ouders	Allergisch eczeem bij een van de ouders
Astma	**2.6 (1.7-4.0)**	1.0(0.7-1.5)	1.0 (0.6-1.6)
Hooikoorts	**2.5 (1.6-4.0)**	**3.6 (2.9-4.6)**	**1.7 (1.1-2.5)**
Allergisch eczeem	**1.5 (1.0-2.2)**	**1.4 (1.1-1.8)**	**3.4 (2.6-4.4)**

Weergegeven zijn de risico's op een allergische ziekte (odds ratio's met 95 % betrouwbaarheidsinterval). Het risico op het hebben van de ziekte wordt vergeleken met kinderen in families die geen eerste graadsfamilieleden met een allergische ziekte hebben. Als het risico 1 is, is dit risico precies gelijk.
Uit: Dold et al. Genetic risk for asthma, allergic rhinitis and atopic dermatitis. Arch Dis Child 1992; 67:1018-1022.

Recente familiestudies bevestigen dit. In een onderzoek in Duitsland werden 6665 families van kinderen tussen de 9 en 11 jaar onderzocht. Astma, hooikoorts en allergisch eczeem bij de ouders gaf een verhoogd risico op dezelfde ziekte bij de kinderen (tabel 1). Ook gaf astma en allergisch eczeem bij één van de ouders een hoger risico op hooikoorts bij de kinderen.[4] Het vóórkomen van een aandoening in bepaalde families kan worden veroorzaakt door gemeenschappelijke erfelijke factoren, maar ook door gemeenschappelijke omgevingsfactoren. Als mensen in een bepaalde omgeving opgroeien en de omgeving bepaalt de aandoening, dan lijkt het immers ook dat de aandoening in bepaalde families voorkomt, net zoals bij erfelijke aandoeningen. Uit tweelingenonderzoek blijkt dat de erfelijke bijdrage aan astma geschat wordt tussen de 36 en 79 %[5]; voor hooikoorts is dit tussen 33 en 82 %[6,7] en voor atopisch eczeem 71 tot 74%.[6] Hieruit blijkt dat zowel erfelijke als omgevingsfactoren een rol spelen bij het optreden van deze allergische ziektes (figuur 1).[8]

Figuur 1. Allergie als uiting van erfelijkheid en omgeving.



Naar Ober C. Genetics of atopy. In: Barnes PJ, Grunstein MM, Leff AR, Woolcock AJ (editors). Asthma. Philadelphia, Lippincott-Raven Publishers, 1997. [8] Weergegeven met toestemming van de uitgever.

Dit proefschrift beschrijft onderzoek onder 200 families met astma (1259 personen). Dit zijn familieleden van 200 mensen met astma, die tussen 1962 en 1975 werden onderzocht in het toenmalig astmacentrum Beatrixoord in Haren. Tussen 1990 en 1999 zijn deze mensen opnieuw uitgenodigd voor onderzoek, waarbij ook partners, kinderen, en eventueel partners van kinderen en kleinkinderen werden onderzocht. Bij alle familieleden werden vragenlijsten afgenomen, de longfunctie werd onderzocht en de overgevoeligheid van de luchtwegen (de bronchiale hyperreactiviteit) werd gemeten door inademing van steeds hogere concentraties histamine. Mensen met astma worden vaak benauwd van al heel lage concentraties van deze stof, terwijl mensen zonder astma hier niet benauwd van worden. Ook werden kenmerken van allergie gemeten: de totale hoeveelheid IgE in het bloed, specifieke IgE antilichamen tegen huisstofmijt, huidtesten, en het totaal aantal eosinofiele granulocyten in het bloed. Dit erfelijkheidsonderzoek werd uitgevoerd in samenwerking met Prof. D.A Meyers en Prof. E.R. Bleecker, Wake Forest University, Winston-Salem, Verenigde Staten.

In dit proefschrift werd onderzocht in hoeverre de erfelijkheid bijdraagt aan deze allergische kenmerken. Onderzoek onder tweelingen toont aan dat astma voor een belangrijk deel erfelijk is, maar dat de omgeving ook meespeelt (deel 1). In ons eigen onderzoek bleek dat de erfelijke bijdrage het grootst was bij totaal IgE in het bloed (55%) en specifiek IgE voor huisstofmijt (57 %); voor specifiek IgE tegen de meest voorkomende allergenen was dit 41%; voor de hoeveelheid eosinofiele granulocyten in het bloed 30 %; voor huidtesten tegen huisstofmijt 29% en tenslotte voor huidtesten tegen de meest voorkomende allergenen 25% (deel drie).
Naast erfelijkheid is ook de omgeving belangrijk bij allergie. In deel twee van dit proefschrift wordt aangetoond dat de kans op allergie kleiner wordt naar mate de grootte van het gezin toeneemt; met andere woorden: hoe meer (oudere) broertjes en zusjes een kind heeft, hoe kleiner de kans op allergie. Het is van belang dat we dit effect konden opsporen bij kinderen van patiënten met (allergisch) astma. Het betekent namelijk dat omgevingsfactoren een belangrijke rol spelen bij kinderen die een hoog risico op allergie hebben omdat het in de familie zit. Dit beschermende effect van de grootte van het gezin zou kunnen berusten op de mogelijkheid dat in grotere gezinnen meer infecties voorkomen, die de ontwikkeling van allergie afremmen. Dit wordt de 'hygiëne hypothese' genoemd. Als we beter leren begrijpen hoe dit effect precies werkt, zouden we dit misschien in de toekomst kunnen gaan gebruiken om allergie te voorkómen bij kinderen van allergische ouders.

Erfelijkheidsonderzoek

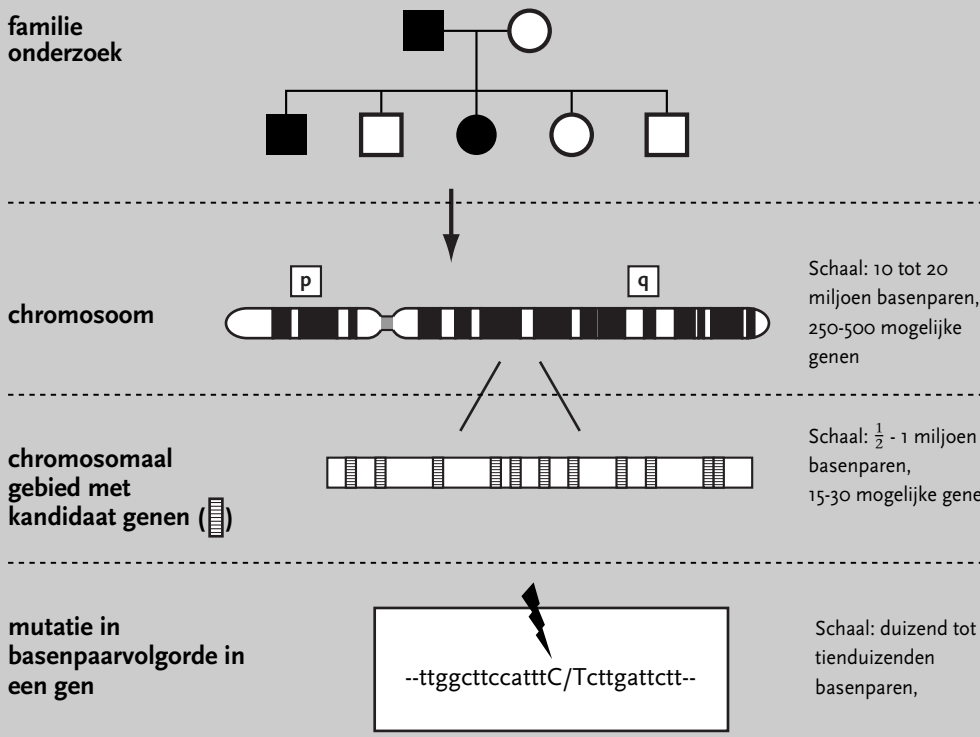
De erfelijke informatie bij de mens bestaat uit 22 paar chromosomen en 1 paar geslachtschromosomen. De korte arm van een chromosoom heet p, de lange arm q (figuur 2). Chromosomen zijn opgebouwd uit lange strengen

basenparen; in totaal zijn er 3 miljard. Een paar procent van deze erfelijke informatie bestaat uit genen. Een gen is een pakketje erfelijke informatie dat de instructie bevat om een eiwit te maken. Volgens recente schattingen heeft de mens circa 30.000 tot 40.000 genen.[9] Een variant in een gen kan leiden tot een verandering in de activiteit of hoeveelheid van het genproduct en zo leiden tot ziekte.[10] De allergische ziekten astma, hooikoorts en atopisch eczeem worden genetisch complexe ziekten genoemd. Deze worden niet door één gen, maar door samenwerking van verschillende genen en omgevingsfactoren veroorzaakt. Tot de genetisch complexe ziekten horen frequente, vaak chronische ziekten zoals astma, hart -en vaatziekte en suikerziekte.

Op zoek naar genen voor allergie op het menselijk genoom

Voor het opsporen van genen voor allergie in de mens bestaan twee strategieën: 'positional cloning' en de kandidaat-gen methode. 'Positional cloning' is gebaseerd op het opsporen van genen met behulp van overervingsanalyses (figuur 2). Allereerst wordt binnen een familie bestudeerd welk chromosomaal gebied samen overerft met de ziekte (linkage-analyse). Daarna begint het tijdrovende proces om binnen dit gebied het verantwoordelijke gen, en de varianten in dit gen op te sporen die de ziekte veroorzaken. Anno 2001 verkeert veel genetisch onderzoek van allergie in dit laatste stadium. Linkage met allergie is gevonden en gerepliceerd op chromosoom 5q, 6p, 7q, 11q en 12q.[5,11] In deel 3 van dit proefschrift wordt linkage analyse van allergie in de 200 Nederlandse families beschreven. Aanwijzingen voor linkage met totaal serum IgE zijn gevonden op chromosomen 5q, 7q, en 12q (hoofdstuk 5). Tevens is linkage-onderzoek verricht van specifiek IgE, huidtesten en het aantal eosinofiele granulocyten in het bloed (hoofdstuk 6). Er werden aanwijzingen gevonden dat de chromosomen 2q, 6p, 11q, 13q, 17q en 22q genen bevatten die verband houden met allergie. Nader onderzoek van het gebied op chromosoom 2 maakte het zeer waarschijnlijk, dat hier een allergie gen ligt (hoofdstuk 7). Het bleek dat in dit gebied op het tweede chromosoom het gen ligt voor CTLA-4 en voor CD28. Dit zijn belangrijke receptoren (ontvangststations) op afweercellen (T-cellen) die mede bepalen hoe sterk de afweerreactie van deze cellen is. Bepaalde varianten in dit CTLA-4 gen kwamen vaker voor bij mensen met allergie en astma vergeleken met niet allergische mensen. Dit gold niet voor CD28. Onze aanwijzingen dat CTLA-4 een allergie-gen is zullen in de toekomst nader onderzocht moeten worden in andere bevolkingen.
Opvallend is dat linkage-onderzoeken in verschillende bevolkingen niet dezelfde chromosomale gebieden vinden. Hier zijn drie verklaringen voor. Ten eerste verschilt de definitie van astma en allergie sterk tussen verschillende studies. Een tweede verklaring is genetische heterogeniteit; dit betekent dat in verschillende populaties andere genen belangrijk zijn. Ten derde zijn sommige studies te klein in omvang om alle linkage resultaten kunnen te bevestigen.

Figuur 2. 'Positional cloning'



Kandidaat-genen voor allergie

Een tweede strategie, de kandidaat-gen methode, heeft meer informatie opgeleverd over mogelijke allergie genen. Een kandidaat gen wordt gekozen omdat het binnen een chromosomaal gebied dat linkage vertoont ligt en/of omdat kennis over de oorzaken van allergie dit gen een waar schijnlijke kandidaat maakt om de ziekte te veroorzaken. Een hogere frequentie van een bepaalde variant in een gen bij een groep patiënten in vergelijking met een groep gezonde mensen betekent een associatie van deze variant met de ziekte. Een associatie is geen bewijs dat het ook de oorzaak van de ziekte is, omdat ook linkage disequilibrium (de veroorza kende variant ligt in erg dicht in de buurt van de onderzochte variant en komt daarom vaak samen voor in de bevolking) en populatie stratificatie (de groepen van patiënten en gezonde controles zijn verschillend samengesteld qua etnische achtergrond) hieraan ten grondslag kan liggen. Voordat geaccepteerd is dat een variant in een gen een oorzaak van allergie is, moet aan vier criteria worden voldaan: (1) Het gen ligt in een chromo-

somaal gebied van linkage; (2) een variant in het gen is geassocieerd met allergie; (3) de variant in het gen verandert de functie of de hoeveelheid van het genproduct en (4) de functie van het gen is biologisch plausibel voor de ontwikkeling van allergie.

In de literatuur verschijnen vele associaties van varianten in kandidaatgenen met allergie. In dit proefschrift wordt kandidaat-gen onderzoek van vijf genen beschreven: Naast CTLA-4 en CD28 zijn dit CD14, IL-13 en de IL4-receptor. Deze laatste drie zullen hieronder verder besproken worden.

CD14

Dit is een receptor (ontvangststation) voor lipopolysachariden, dit zijn stoffen die zich bevinden in de wanden van bacteriën. CD14 bevindt zich op de buitenkant van verschillende bloedcellen die belangrijk zijn bij de afweer, deze bloedcellen worden monocyten, macrofagen en neutrofiele granulocyten genoemd. Ook is er een oplosbare vorm in het bloed. CD14 werd als kandidaat gen voor allergie onderzocht vanuit de hygiëne hypothese, die veronderstelt dat infecties de ontwikkeling van allergie onderdrukken. Men onderzocht het promoter gebied, dit is de DNA volgorde die voorafgaat aan het gen zelf en die de mate van de productie van het gen reguleert. In dit promoter gebied vond men een variant die was geassocieerd met het aantal positieve huidtesten en een hoog totaal serum IgE spiegel in een groep allergische kinderen in de Verenigde Staten.[14] Deze invloed van CD14 op de ernst van allergie werd bevestigd in ons onderzoek in hoofdstuk 8.[15] Het bleek dat mensen met twee C varianten op plaats –159 in dit gen een ernstiger vorm van allergie hadden dan mensen met twee T varianten. Bovendien hadden ze vaker hooikoorts en neusallergie.

Interleukine 4

Interleukine 4 (IL-4) is een eiwit en het speelt een belangrijke rol bij de productie van IgE. Allereerst zet IL-4 witte bloedcellen, B cellen, aan tot het maken van IgE. Daarnaast beïnvloedt IL-4 de afweerreactie zo, dat er meer cellen komen die allergie bevorderen, deze afweercellen worden Th2 lymfocyten genoemd. Th2 lymfocyten kenmerken zich door de productie van signaal stoffen die ontsteking bevorderen, zoals IL-4, IL-5 en IL-13. In de promoter regio van het IL-4 gen ligt een variant die in sommige studies een verband heeft met de totale hoeveelheid IgE in het bloed. Deze associatie is echter niet aanwezig in alle bevolkingsgroepen die onderzocht zijn, getuige het verschijnen van veel studies met een negatief resultaat.[5]

Interleukine 13

Dit kan evenals IL-4 B-cellen aanzetten tot IgE productie. Daarnaast suggereert onderzoek in een muismodel voor astma dat IL-13 ook een belang rijke rol speelt in de aanzet van ontstekingsreacties in de luchtwegen van astmapatiënten.[16] Het gen van IL-13 kent één variant die leidt tot een aminozuur volgorde verandering en verschillende varianten in het promo

ter gebied. Het afgelopen jaar verschenen vier studies die een rol voor een of meer varianten in dit gen beschrijven met allergisch astma[17,18], totaal IgE in het bloed[19], en allergisch eczeem.[20] Tevens toonde Van der Pouw Kraan et al. aan dat één van de promoter varianten mogelijk de hoeveelheid IL-13 beïnvloedt.[17] In hoofdstuk 9 van dit proefschrift wordt een nadere analyse van IL-13 besproken. Allereerst is het gen dat voor IL-13 codeert geanalyseerd op de aanwezigheid van andere varianten. Er werden een aantal nieuwe varianten gevonden. Vervolgens werd in ons onderzoek aangetoond dat bepaalde varianten in het IL-13 gen vaker voorkomen bij mensen met astma en allergie vergeleken met mensen zonder astma. Dit is een sterke aanwijzing dat IL-13 belangrijk is voor de ontwikkeling van astma.

De α- keten van de IL-4 receptor (IL4Rα)

Dezez vormt samen met een andere IL-4 receptor keten, de IL-4Rγ keten, het ontvangststation voor IL-4. Samen met de IL-13 receptor α1 of α2 keten vormt dezelfde IL4Rα de receptor voor IL-13. Verschillende varianten beïnvloeden mogelijk de sterkte van het signaaloverbrenging van deze receptor. Deze varianten zijn alleen of in combinatie bestudeerd en bleken geassocieerd met allergisch astma, totaal en specifiek serum IgE.[21] In hoofdstuk 10 wordt de associatie van allergie met een aantal varianten in het IL-4R gen beschreven. Tevens bleek dat mensen die zowel drager zijn van de astma variant in IL-13 als de allergie varianten in IL-4R maar liefst een vijf keer grotere kans te hebben om astma te krijgen in vergelijking met mensen die deze varianten niet dragen. Dit vormt een sterke aanwijzingen voor de samenwerking van deze twee genen in de ontwikkeling van astma. Op dit moment worden medicijnen ontwikkeld dit IL4/IL13-IL4R pad selectief te remmen. We bevelen daarom aan het effect van deze nieuwe medicijnen allereerst te bestuderen bij mensen die drager zijn van deze varianten in het IL-4R en IL-13 gen. Het zou kunnen dat deze mensen hier bij uitstek baat bij hebben.

Functie van allergie en astma genen

De β_2-adrenerge receptor is het ontvangststation voor veel bij astma gebruikte luchtwegverwijders, de bèta mimetica zoals salbutamol. Er zijn coderende varianten in dit gen bekend, die mede het korte en lange termijn effect van deze medicijnen lijken te bepalen. In deel 5 van dit proefschrift wordt een onderzoek beschreven naar de mogelijke functie van varianten in de β_2-adrenerge receptor. Dit onderzoek heeft als doel de werking te vinden van genen die eerder een associatie vertoonden. Dit onderzoek werd uitgevoerd samen met de Univerisiteit van Nottingham (prof I.P. Hall) en de Unversiteit van Aberdeen (prof B. Lipworth). Er werden een aantal nieuwe promoter varianten bestudeerd, die vaak bleken voor te komen met de varianten in de receptor. De aanwezigheid van deze varianten maakte niet uit voor het aantal β-receptoren op bloedcellen en ook niet voor de mate waarin deze cellen een signaal-stof (cAMP) produceren na stimulering van deze receptoren.

Genetisch onderzoek van allergie bevindt zich momenteel in de fase van wetenschappelijk onderzoek en heeft nog niet de weg gevonden naar de spreekkamer. Wat zijn mogelijkheden van erfelijkheidsonderzoek voor diagnostiek en behandeling in de praktijk?

Diagnostiek

Het is de vraag of genetisch onderzoek een plaats zal krijgen in de diagnostiek van allergische ziektes. Omdat allergie en astma veel voorkomen in de bevolking mag men verwachten dat ook de genen voor deze ziekten veel voorkomen. Door de hoge frequentie van een risico-variant in de bevolking (bij voorbeeld 30%) kan een bepaalde genetische variant sterk bijdragen aan de ziekte op bevolkingsniveau. Als het hebben van deze genvariant een twee keer hogere kans op de ziekte geeft vergeleken met mensen die deze variant niet dragen, dan kan deze genvariant 23.1 % van de ziekte op bevolkingsniveau verklaren. Zal de vaststelling van deze variant ook kunnen helpen bij de de diagnose van astma op individueel niveau? In hetzelfde voorbeeld bedraagt de kans dat iemand de ziekte krijgt bij het hebben van deze genvariant slechts 7.7%.[24] De diagnostische bijdrage van varianten met een hoge frequentie in de bevolking en een gering risico op ziekte zal dus laag zijn voor het vaststellen van een ziekte op individueel niveau. Of het vaststellen van meerdere genvarianten, zoals in ons onderzoek werden gevonden voor IL-13 en IL-4R, voldoende voorspellend zal zijn voor het vaststellen van een risicogroep en om bij deze groepen vervolgens preventieve maatregelen ten nemen is vooralsnog onduidelijk.

Behandeling

De hoofdreden van de speurtocht naar genen voor ziekten is het vinden van erfelijke oorzaken die bijdragen aan een beter begrip van de oorsprong van de ziekte. Omdat de functie van de meerderheid van de genen niet bekend is, lijkt dit een aantrekkelijke optie. Een recent onderzoek naar een gen dat suikerziekte zou kunnen veroorzaken illustreert dit. Dit is de eerste publicatie over een gen dat is gevonden met behulp van 'positional cloning' en –indien gerepliceerd- opent deze ontdekking een nieuwe rich ting in het onderzoek naar de oorzaken van diabetes mellitus type II.[25] Met de voltooiing van het Human Genome Project, nu gepland in 2003, zal in de komende jaren de basenpaarvolgorde van het menselijk genoom bekend worden, waarna de grote uitdaging volgt om functie van genen en de rol bij ziekte op te helderen.

Een tweede veld van onderzoek vormt de farmacogenetica, dat de rol van de erfelijkheid bestudeert in de reactie op medicijnen. Dit gaat zowel om het vóórkomen van bepaalde genetische varianten bij mensen die niet beter worden van een behandeling, als ook het bestuderen van risico-genen voor het krijgen van ernstige bijwerkingen op medicijnen. Op het gebied van astma zijn inmiddels verschillende genen bestudeerd voor wat betreft

hun rol in de reactie op behandeling: de β_2-adrenerge receptor[26] en het 5-lipoxygenase gen (ALOX-5).[27] In dit laatste onderzoek bleek dat patiënten met astma die drager waren van een mutatie in dit ALOX-5 gen niet verbeterden in longfunctie na gebruik van een experimentele 5-lipoxygenase remmer. Dit in tegenstelling tot patiënten met astma met de normale variant in dit gen, die na 64 dagen gebruik gemiddeld 18.8 % verbeterden met de FEV_1, dit is de hoeveelheid lucht die men in 1 seconde kan uitblazen en is een maat om luchtwegvernauwing aan te geven.[27] Indien nieuw farmacogenetisch onderzoek, bijvoorbeeld naar resistentie tegen ontstekingsremmers (corticosteroïden) laat zien dat genetische variatie fors bijdraagt aan reactie op behandeling, zal dit snel zijn weg kunnen vinden naar de klinische praktijk.[28]
Tenslotte lijkt gentherapie nog ver buiten beeld. De reden hiervoor is niet alleen de methodologische problemen van gentherapie (lage effectiviteit, soms fatale bijwerkingen), maar ook het feit dat bij complexe genetische aandoeningen meerdere genen en omgevingsfactoren samenwerken. Dit maakt toepassing van gentherapie onwaarschijnlijk.[29]

Conclusies

Bij de ontwikkeling van allergische ziekten spelen genetische factoren een belangrijke rol, in samenwerking met omgevingsfactoren. Genetisch onderzoek heeft tot nu toe wel gebieden op het menselijk genoom geïdentificeerd die zeer waarschijnlijk allergie genen bevatten (chromosomen 2, 5, 6, 7, 11 en 12), maar het is nog niet duidelijk welke genen dit precies zijn. Uit kandidaat-gen onderzoek volgt dat varianten in de genen die coderen voor CTLA-4, IL-13, IL-4Rα, CD14 wellicht van belang zijn. Toch zal hier nog veel onderzoek naar moeten gebeuren. Genetisch onderzoek van astma en allergie heeft nog niet de weg gevonden naar de klinische praktijk. Voordat dit het geval is, moeten eerst nog een aantal belangrijke vragen beantwoord worden:

- In welke mate draagt een bepaalde variant in een gen bij tot allergische ziekten? Zijn er verschillende genen voor de verschillende allergische ziekten? Is er een toepassing in diagnostiek op individueel of groepsniveau?
- Is er een samenwerking van een bepaalde variant in een gen met andere genetische of omgevingsfactoren?
- Is er een relatie tussen bepaalde varianten in een gen met de reactie op behandeling?

In de komende jaren zal binnen dit onderzoeksgebied een grote uitdaging zijn om deze vragen te ontrafelen, zodat op den duur toepassingen van erfelijkheidsonderzoek beschikbaar kunnen komen voor de patiënt met een allergische ziekte.

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